UNIVERSIDADE DO ALGARVE
MESTRADO EM CIÊNCIAS BIOMÉDICAS

MORPHOLOGIC AND MOLECULAR RESPONSES OF DEVELOPING RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) TO DIETARY MINERALS

NÁDIA SILVA Nº 9669

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DISSERTAÇÃO DE MESTRADO ORIENTADA POR:

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The contents of this dissertation are of exclusive responsibility of the author.

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ABSTRACT

Calcium (Ca) and phosphorus (P) play a critical role in diverse biological processes, and their regulation is essential for organism health. In the present study, the adaptive response to Ca and P dietary availability in trout (*Oncorhynchus mykiss*) was evaluated taking an integrative approach. The impact of dietary restriction in Ca and P on skeletal ontogeny, morphology and density was assessed. The expression of previously known Pi responsive genes, along with calciotropic hormones STC, CT and extracellular matrix proteins (osteonectin and osteopontin) was evaluated by RT-qPCR. The activity of the ultimobrachial gland and corpuscles of Stannius was determined by morphometry after immunohistochemistry for calcitonin (CT) and stanniocalcin (STC) respectively. P restricted individuals showed decreased whole body P and Ca content throughout the experiment. The restriction of dietary P led to delayed skeletal ontogeny which affected the mineralization level of both endochondral and dermal bones, and was associated with an increase in the incidence of skeletal malformation. Low Ca and increased P in the diet also delayed skeletal development at D11 but this effect was transient. High dietary P caused increased plasma levels of P by D11 and a cumulative mortality rate of 90% by the end of the experiment. These individuals had denser bones and a lower incidence of malformations, possibly due to the effect of "survival of the fittest". Of the previously identified molecular markers of P deficiency in trout S10011A was found to be significantly downregulated in P deficient trout fry, while INaPiIIb was dowregulated in trout fry on low Ca and high P diets. The calciotropic hormone STC was upregulated in trout fry on low P diets and CT was downregulated in trout fry on high P and low Ca diets. OSN expression was also downregulated in the high P group. The study indicates that modifications in the dietary Ca and P during development significantly modify the ontogeny and density of the skeleton. The availability of Ca and P also led to modification in endocrine factors presumably to ensure maintenance of mineral homeostasis. It seems likely that changes in hormone production may be responsible for changes in transcript abundance of specific transporters/binding proteins of Ca and P although in the present study it was not possible to test this hypothesis.

KEYWORDS: Skeletal development; Dietary minerals; P deficiency; Gene expression; Calcium; Phosphorus; Mineral metabolism

RESUMO

OBJECTIVO: O cálcio (Ca) e o fósforo (P) desempenham um papel fundamental em diversos processos biológicos, e a sua regulação é essencial para a saúde do organismo. No presente estudo, o objectivo foi o de estabelecer de que modo a quantidade de fósforo e cálcio na dieta influenciam a ontogenia esquelética mas também em parâmetros bioquímicos, moleculares e endócrinos em larvas de truta (Oncorhynchus mykiss).

MÉTODOS: A resposta adaptativa á disponibilidade de Ca^{2+} e de P na dieta foi avaliada tendo em conta o efeito sobre a composição corporal, nomeadamente conteúdo em cinza e matéria seca, na ontogenia do esqueleto. Esta foi analisada pelo método de coloração cartilagem-osso seguida de contagem do número de estruturas presentes e classificação do seu estado de ossificação. Em indivíduos juvenis utilizou-se RX para avaliação da densidade óssea, características morfométricas da coluna vertebral e também a incidência de anomalias. A variação na expressão de genes previamente reconhecidos em diversos trabalhos como tendo resposta ao Pi na dieta foi analisada por RT-QPCR. Estes incluíram transportadores de fósforo MtPi, INaPiIIb, mas também Meprin 1A, Vitamin D receptor e S10011A. A expressão de hormonas calciotrópicas: calcitonina (CT) e staniocalcina (STC) cuja função em peixes ainda levanta algumas questões foi também analisada por RT-QPCR juntamente com a análise do estado de actividade das glândulas calciotrópicas: Glândula Ultimobranquial e Corpúsculos de Stannius, produtoras respectivamente de CT e STC. Esta análise foi efectuada por morfometria, utilizando para identificação da localização das glândulas, imunohistoquímica para detecção de STC e CT. Com vista a relacionar o efeito de Pi e Ca^{2+} na dieta sobre formação do esqueleto com proteínas pertencentes à matriz extracelular óssea a expressão dos genes da osteonectina e osteopontina foi também avaliada por RT-QPCR.

RESULTADOS: Indivíduos que receberam a dieta restrita em P apresentaram uma diminuição no conteúdo corporal de P e Ca^{2+} durante toda a duração da experiência. A restrição dietética de Pi levou a um atraso generalizado na ontogenia do esqueleto, que afectou também a mineralização de estruturas de origem endocondral e dermal, e levou a uma elevada incidência de malformações esqueléticas. A densidade óssea determinada por RX em indivíduos juvenis alimentados com pouco Pi era significativamente inferior aos restantes grupos, confirmando um efeito osteoporótico da dieta com pouco P. Nos indivíduos alimentados com restrição de Ca^{2+} e excesso Pi também houve um ligeiro

atraso no desenvolvimento esquelético a D11 mas este efeito foi transiente e em indivíduos juvenis a densidade óssea era semelhante ao grupo controlo, embora a incidência de anomalias fosse mais elevada. A dieta que apresentava excesso de Pi causou o aumento dos níveis plasmáticos de Pi detectado ao D11 e originou durante o período da experiencia 90% mortalidade. No entanto estes indivíduos apresentaram uma densidade óssea superior juntamente com a menor incidência de malformações relativamente aos restantes grupos na fase juvenil, podendo isto ficar a dever-se à selecção de indivíduos provocada pela mortalidade. Dos marcadores de deficiência de Pi previamente identificados em trutas adultas, apenas o gene S10011A mostrou possuir uma expressão decrescida em resposta a este desafio, enquanto o iNaPiIIb se encontrava com expressão diminuída nos grupos alimentados com excesso de fósforo e restrição de cálcio. A hormona calciotrópica STC sofreu um aumento de expressão nos indivíduos LP e a CT sofreu um decréscimo de expressão nos grupos alimentados com excesso de fósforo e restrição de cálcio. A expressão de OSN também se encontrava diminuída no grupo alimentado com excesso de fósforo. Os dados recolhidos desta experiência indicam que a resposta adaptativa á restrição de minerais na dieta, como se pode ver pela modificação da transcrição genética pode ser diferente em larvas e trutas adultas. O estudo indica que a expressão de genes marcadores encontrados em peixes adultos não pode ser transposta directamente para análise de indivíduos em desenvolvimento o que pode estar relacionado com uma vasta gama de factores desde o estado de maturação do tecido a fisiologia dos peixes jovens, os requerimentos nutricionais etc. No entanto este estudo indica que modificações dos níveis de Ca e P na dieta durante o desenvolvimento alteram significativamente a ontogenia e densidade do esqueleto. A disponibilidade em P e Ca levou também á modificação de factores endócrinos presumivelmente de forma a assegurar a manutenção da homeostasia mineral. Parece provável que alterações na produção hormonal, possam ser responsáveis pela alteração na abundância de transcritos de transportadores e proteínas de ligação ao Ca e P, embora no presente estudo não tenha sido possível testar esta hipótese

PALAVRAS-CHAVE: Desenvolvimento esquelético, Conteúdo mineral na dieta; Deficiência de fósforo; Expressão genética; Cálcio; Fósforo; Metabolismo mineral.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 5

TABLE OF ABBREVIATIONS

C	CONTROL
Ca	CALCIUM
CaP	CALCIUM PHOSPHATE
CaSR	CALCIUM-SENSING RECEPTOR
CS	CORPUSCLES OF STANNIUS
CT	CALCITONIN
dpf	DAYS POST FERTILIZATION
dph	DAYS POST HATCHING
ECM	EXTRACELLULAR MATRIX
HP	HIGH PHOSPHORUS
I-NaPiIIb	INTESTINAL PHOSPHORUS/SODIUM CO-TRANSPORTER IIB
LC	LOW CALCIUM
LP	LOW PHOSPHORUS
MEP	MEPRIN 1A
mRNA	MESSENGER RIBONUCLEIC ACID
mtPi	MITOCHONDRIAL INORGANIC PHOSPHATE CARRIER
OSC	OSTEOCALCIN
OSN	OSTEONECTIN
OSP	OSTEOPONTIN
P	PHOSPHORUS
PCNaPiIIb	PYLORIC CAECA PHOSPHORUS/SODIUM CO-TRANSPORTER IIB
PTH	PARATHYROID HORMONE
PTHrP	PARATHYROID HORMONE RELATED PEPTIDE
RT-QPCR	REVERSE TRANSCRIPTASE -QUANTITATIVE POLYMERASE CHAIN REACTION
S100	S100 CALCIUM BINDING PROTEIN
SL	STANDARD LENGTH
STC	STANNIOCALCIN
UBG	ULTIMOBRANCHIAL GLAND
VDR	VITAMIN D RECEPTOR
Vit D	VITAMIN D

PART 1

MORPHOLOGIC AND MOLECULAR RESPONSES OF DEVELOPING RAINBOW TROUT TO DIETARY MINERALS

1.1 General Introduction

Skeleton is made of bone and cartilage. The fact that bones are the most inert and lasting of vertebrate physical remains, makes it amazing how dynamic and regulated they are during life (DeWitt 2003). The skeleton provides mechanical support and mobility to the body by providing attachments for muscles, ligaments, and tendons, protection of vital organs, hematopoiesis in bone marrow and regulation of blood calcium levels (Karsenty et al. 2002; Cohen 2006). Cartilage is an avascular supporting and articular skeletal tissue and it functions as the primary endoskeletal support in vertebrate embryos (Hall and Witten 2007). All these functions require the maintenance of an adequate bone shape and bone density achieved by the ongoing dynamic process of bone formation and resorption - called bone remodeling, which is constantly occurring on a microscopic scale throughout our bodies and leads to 10% of bone replacement each year giving a complete bone mass renewal every 10 years (Cohen 2006). The balance of bone formation and resorption is achieved by interplay of mineral deposition and resorption by specialized bone cells. An imbalance in this system leads to a number of diseases of bone and cartilage. Bone mass is governed by a complex interaction of genetic, age-related, hormonal, nutritional, environmental and life style factors in the regulation of systemic calcium and phosphate homeostasis and local bone remodeling processes (Pietschmann and Peterlik 1999). There is therefore a need for a better understanding of both the fundamentals of skeletal biology and related pathologies (Karsenty et al. 2002), in order to accomplish that it is fundamental to expand the knowledge of mineral metabolism mechanisms.

Bone and cartilage composition

Bone and cartilage constituents are resumed in Table 1.1, both are formed by cells, mineralized matrix and organic matrix with different compositions. The organic bone matrix is mostly comprised of type I collagen. The three major noncollagenous proteins of bone matrix are osteocalcin (OSC) a vitamin K dependent, Ca^{2+} binding protein that recruits osteoclasts or osteoclast precursors to bone for resorption (Hauschka et al. 1989; Nakamura et al. 2009); osteopontin (OSP), which is a glicosylated phosphoprotein that enhances survival and migration of osteogenic cells. The level of

phosphorylation of OSP apparently has an impact on its function, when 40% of its phosphorylation sites are filled mineralization does not occur, when 95% of its sites are filled, it appears to promote hydroxyapatite formation and may act as an anchor of osteoclasts to bone (Reinholt et al. 1990; Gericke et al. 2005); and osteonectin (OSN), which is the extracellular matrix protein that links collagen to hydroxyapatite, serves as a nucleus for mineralization, and regulates the formation and growth of hydroxyapatite crystals (Nomura et al. 1988; Termine et al. 1981; Yagami et al. 1999; Hall 2005; Hall and Witten 2007)

The mineralized matrix of bone is constituted by hydroxyapatite, an hydroxylated polymer of calcium phosphate [$Ca_{10}(PO_4)6(OH)_2$]. Cartilage is formed by an hydrated extracellular matrix mostly comprised of collagen 2 and proteoglycans and may or may not be mineralized depending on the cartilage type (Hall 2005; Cohen 2006; Hall and Witten 2007).

Table 1.1 - Components of bone and cartilage. Adapted from (Cohen 2006).

	BONE	**CARTILAGE**
CELLS	Osteoblasts Osteocytes Osteoclasts	Chondroblasts Chondrocytes
MINERALIZED MATRIX	$Ca_{10}(PO_4)6(OH)_2$ Carbonate, citrate, fluoride, chloride, sodium, magnesium, potassium, strontium	$Ca_{10}(PO_4)6(OH)_2$ Hyperhydrated structure Water (80%)
ORGANIC MATRIX	Type I collagen (88%) Other proteins (10%) Osteocalcin Osteonectin Phosphoproteins Lipids and glycosaminoglycans (2%)	Type II collagen (major component) Proteoglycans (major component) Other proteins (trace amounts) Neutral lipids Phospholipids Lysozyme Glycoproteins

Three distinctly different cell types (Table 1.1) can be found within bone: the matrix-producing osteoblast, the tissue-resorbing osteoclast, and the osteocyte, which accounts for 90% of all cells in the adult skeleton. Osteocytes can be viewed as highly specialized and fully differentiated osteoblasts (Sommerfeldt et al. 2001). Osteoclasts are multinuclear cells which adhere to bone matrix and secrete acid and lytic enzymes that degrade both the mineral and organic bone matrix (Boyle et al. 2003). Within cartilage are found chondrocytes at various stages of development: chondroblasts, mature chondrocytes and hypertrophic chondrocytes, depending on the status of cartilage

development and also localization within cartilage. Osteoblasts are the main cell type responsible for the production of the characteristic extracellular matrix (ECM) and mineralization of the bone matrix. The mineralized bone matrix gives strength to the skeleton and provides a structural framework for mineralization and modulates functions such as cell adhesion/migration and growth factor signaling. Osteoblasts play a central role in these processes and also participate in regulating the differentiation of osteoclasts, the bone-resorbing cells (Nakashima and de Crombrugghe 2003).

Formation of bone

Bone formation in tetrapods occurs through two distinct developmental processes, intramembranous ossification and endochondral ossification (Figure 1.1); they both result from a sequence of events involving epithelial–mesenchymal interaction, condensation, and differentiation. Intramembranous ossification occurs mainly in the craniofacial bones and the lateral part of clavicles, osteoblasts differentiate directly from mesenchymal condensations. In endochondral ossification which occurs in long bones, the basal part of the skull, vertebrae, ribs and medial part of the clavicles, an intermediate step is involved where cartilaginous structures serve as a template for the future bony elements. In some of these elements cartilage persists as part of the definitive structure (Nakashima et al. 2003; Hall 2005; Hall and Witten 2007). The gross structure of adult mammalian bone is composed of two types of bone: cortical which forms the outer layer and is hard and serves as a placement for muscle attachment; and cancellous or trabecular bone which constitutes the inner portion and is in contact with the bone marrow (Hillier et al. 2007).

Bone formation is a tightly regulated process which is characterized by a sequence of events starting by the commitment of osteoprogenitor cells, their differentiation into pre-osteoblasts and mature osteoblasts whose function is to synthesize the bone matrix (Marie 2008). The first bone matrix deposited is unmineralized and is known as osteoid. Bone mineralization starts by the production of osteoid by osteoblasts, its subsequent impregnation with hydroxyapatite in a 2 step process (Figure 1.2) to form the mineralized tissue. Physiological mineralization is a highly regulated process that takes place during the formation, development, remodeling and repair of skeletal tissues; it is complex and involves several components. Mineralization takes place within the

extracellular matrix niche, and involves primarily the formation and release by osteoblasts of cytoplasmic membrane-bound matrix vesicles, near the osteoid-osteoblast interface (Anderson 1995; Anderson et al. 2005; Stewart et al. 2006; Xiao et al. 2007).

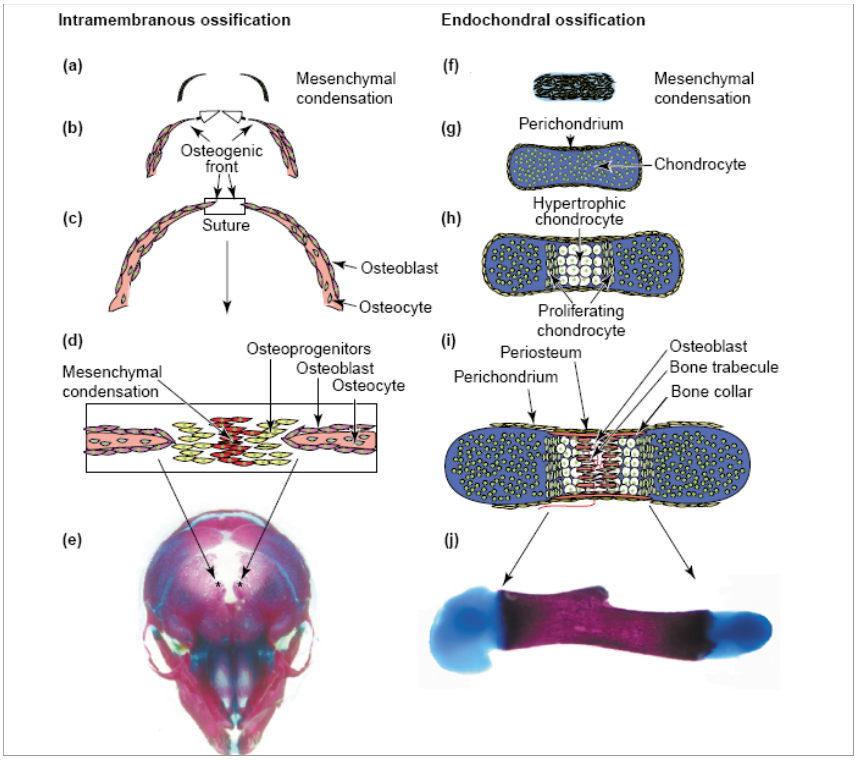

Figure. 1. 1 - Process of bone formation in mice. (a–d) In intramembranous ossification bones are formed directly from condensations of mesenchymal cells. (a) formation of frontal bones starts with mesenchymal condensations on the lateral side of the head. (b) From the mesenchymal condensation, the cellular mass with osteogenic activity spreads upward toward the top of the skull (arrows). Osteoblasts differentiate from this mass and produce bone matrix. (c,d) At the extremities cells differentiate into osteoprogenitor cells which give rise to osteoblasts that produce bone-matrix molecules. Osteoblasts will differentiate into osteocytes, which become embedded within the bone matrix. (e) A schematic frontal view of a mouse skull stained with alizarin red, for bone and alcian blue, for cartilage. Asterisks indicate osteogenic fronts of frontal bones. (f) Development of endochondral bones also starts with the formation of mesenchymal condensations. (g) Cells in these sites give rise to chondrocytes whereas cells at the periphery of the condensations form a perichondrium, which is a dense irregular connective tissue. Centrally localized chondrocytes proliferate actively and differentiate into hypertrophic chondrocytes when the cell cycle comes to a halt. The sequential differentiation of chondrocytes establishes a unique cellular organization that constitutes the epiphyseal growth plate. At the distal end of the epiphyseal growth plate, hypertrophic chondrocytes further mineralize their own cartilaginous matrix. In parallel to this multistep differentiation pathway, cells from a thin layer of mesenchymal cells, called the periosteum, that envelops the cartilage matrix and contains osteoprogenitor cells, invade the zone of hypertrophic chondrocytes together with blood vessels and osteoclasts. Osteoclasts start degrading this mineralized cartilaginous matrix and this semi-degraded matrix serves as a template for the deposition of bone matrix by osteoblasts (i). Endochondral bone is surrounded by cortical bone (the bone collar) that surrounds the marrow cavity and growth plate. The cortical bone is contiguous with

the perichondrium. (j) A mouse humerus stained with alizarin red and alcian blue. Taken from (Nakashima et al. 2003)

These matrix vesicles contain a variety of substances, including annexin V (on the inner membrane surface), alkaline phosphatase (GPI-linked), calbindin-D_{9K}, pyrophosphatases, carbonic anhydrase, AMPase, BSP-1, osteonectin, and osteocalcin. The matrix vesicle membrane is enriched in cholesterol, phosphatidylserine, and sphingomyelin. Ion channels and transporters present in MV membrane act for Ca^{2+} and Pi uptakes into these organelles by annexin and sodium-phosphate co-transporter 3 (NaPi3) respectively and promote the accumulation of Ca^{2+} and Pi forming non-crystalline, amorphous calcium phosphate (CaP). This compound is converted into octa CaP crystals and with more Ca^{2+} and water from the insoluble hydroxyapatite crystals $Ca_{10}(PO_4)6(OH)_2$ in their lumen.

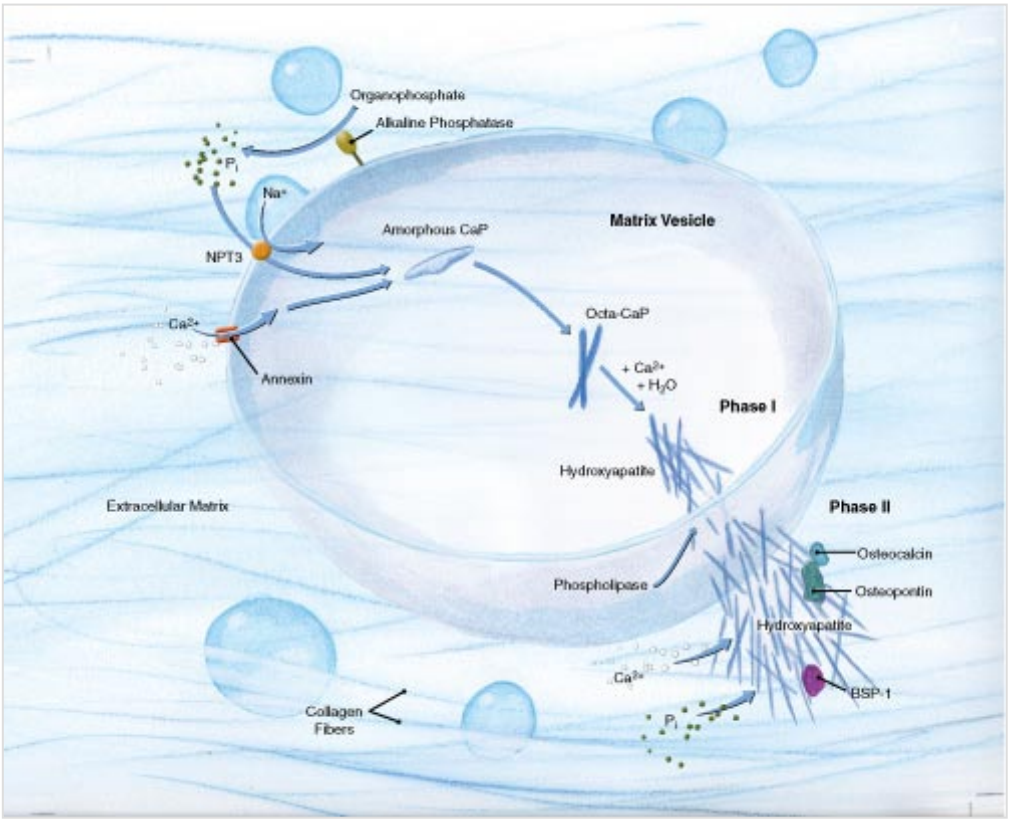

Figure 1.2 - Mechanism of hydroxiapatite formation. Taken from (R&DSystems 2007).

In a second phase of the mineralization process, the breakdown of the vesicle membranes by phospholipases release the hydroxiapatite from the vesicle membrane and the levels of extra-vesicular Ca^{2+}, P_i, and H^+, as well as Ca^{2+}-binding proteins,

bone-sialo protein-1, osteocalcin, and osteopontin, secreted also by the osteoblasts regulate the continued nucleation of hydroxyapatite crystals and consequent propagation of mineralization of the ECM. Factors that regulate the continuing growth of the hydroxyapatite include Ca^{2+} and P_i concentrations, local pH, and the presence of non-collagenous matrix proteins (Anderson 1995; Anderson et al. 2005; Stewart et al. 2006; Xiao et al. 2007).

Skeletal cells

Osteoblast commitment, differentiation and function are governed by several transcription factors, resulting in expression of phenotypic genes and acquisition of the osteoblast phenotype that will ultimately define the formation of bone. They share the same mesenchymal cell precursor as chondrocytes and adipocytes (Figure 1.3), whilst osteoclasts are derived from hematopoietic precursors located in the monocytic fraction of the bone marrow (Sommerfeldt et al. 2001).

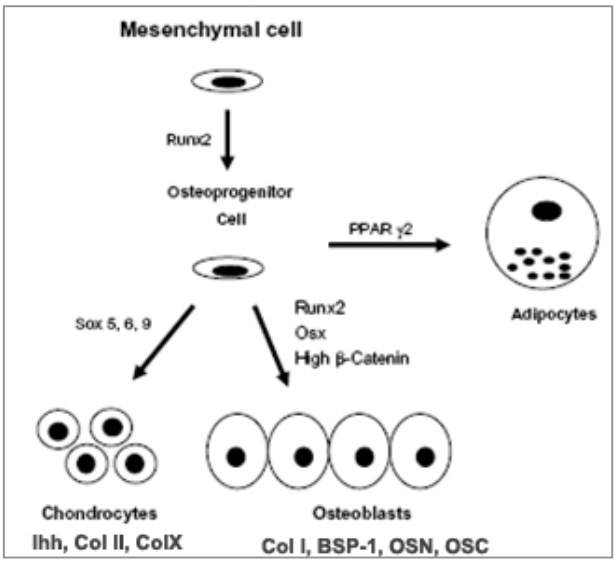

Figure 1.3 – Transcription factors controlling mesenchymal cell differentiation. Osteoblasts and chondrocytes are both derived from the same mesenchymal progenitor, as are pre-adipocytes, through differential transcriptional control. RUNx2 is essential for osteoprogenitor lineage differentiation, which can then give rise to chondrocytes through the action of Sox 5, 6 and 9, or osteoblasts trough the action of RunX2, Osirix (Osx) and high levels of β-Catenin. The osteoprogenitor cell can also give rise to adipocytes through the action of PPARγ2 transcription factor. Mature chondrocytes secrete Indian hedgehog (Ihh), collagen 2 (Col II) and at a later stage of development, hypertrophy the secrete collagen 10 (Col X). Mature osteoblaste secrete collagen 1 (Col I), bone-sialo protein (BSP-1), osteonectin (OSN) and osteocalcin (OSC) (Nakashima et al. 2003; Franz-Odendaal et al. 2006; Marie 2008).

Type I collagen and alkaline phosphatase are molecular markers for the early stage of osteoblast differentiation whilst osteocalcin and mineralization of the extracellular matrix are molecular markers for the late stage of osteoblast differentiation. Other important markers are osteonectin, osteopontin, and bone sialoprotein but numerous other molecular markers have been identified for the different cells and stages of skeletal development (Nakashima et al. 2003; Cohen 2006; Franz-Odendaal et al. 2006).

Hormonal factors such as parathyroid hormone (PTH), estrogens, glucocorticoids and vitamin D control bone formation by regulating the expression of transcription factors in osteoblasts. PTH promotes bone formation in part through phosphorylation and activation of Runx2, resulting in activation of osteoblast genes. Also, PTH inhibits Runx2 degradation by the proteasome. Additionally, PTH increases Osx and reduces PPARγ2 expression in osteoprogenitor cells, resulting in lineage determination towards osteoblasts. Another regulatory action of PTH is to promote β-Catenin pathway which controls osteoblast survival (Marie 2008).

Fish skeleton

Morphologically, fish skeleton comprises dermal and endochondral bones and scales. However, fishes bones do not posses bone marrow (Lall and Lewis-McCrea 2007). There are two types of bones in teleosts, cellular and acellular. Cellular bones are confined to only a few groups of fish, e.g., Salmonidae, Cyprinadae, and Clupeidae. The skeletal tissue of higher orders of teleost fish such as Perciformes is unique among vertebrates in that they are acellular and lack osteocyte cells (Hall 2005; Lall and Lewis-McCrea 2007).

The skeletal ontogeny of teleosts has been described for a number of species (Faustino and Power 1998; Faustino and Power 1999; Koumoundouros et al. 1999; Faustino and Power 2001; Bird and Mabee 2003; Campinho et al. 2004; Estêvão et al. 2005; Estêvão et al. 2009). Skeletal development in bony fish follows the pattern that has been conserved during vertebrate evolution (Lall and Lewis-McCrea 2007) and similarities at both the cellular and developmental level support the suggestion that important regulatory mechanisms implicated in bone homeostasis are conserved between fish and higher vertebrates (Renn et al. 2006). Furthermore alkaline phosphatase is an accepted marker for osteoblasts of teleosts and mammals (Aubin and Liu 1996; Franz-Odendaal

et al. 2006; Witten and Huysseune 2007) and it has been shown that both osteonectin and osteocalcin are expressed in teleosts ands that bone-derived osteoblastic cell lines from sea bream form calcium phosphate crystals similar to hydroxyapatite (Aubin and Liu 1996; Pombinho et al. 2004; Redruello et al. 2005). Therefore many of the molecular markers for endochondral and intramembranous ossification identified in mammals (Franz-Odendaal et al. 2006) have also been found in fish (Fisher and DeGeorge 1967; Renn et al. 2006; Gorman and Breden 2007)

Nutrition and skeleton formation

The effect of dietary factors and nutrients on the skeleton is well studied in mammals. A high level of fiber impairs calcium, lipid and fat-soluble nutrient absorption, isoflavonoids cause decreased bone resorption as a consequence of their estrogen like properties. Promotion of bone formation and mineralization can be achieved by vitamins (A, C, E and K) and minerals (calcium, phosphorus, boron, zinc, copper, silicon, vanadium, selenium, manganese, strontium and fluoride). It is known that excessive intake of vitamin A and some minerals (strontium, lithium, aluminum, iron, molybdenum, cadmium, tin and lead) has a harmful effect on skeletal tissue metabolism (Beattie et al. 1992; Wallach 2002). The growth of the human skeleton is frequently limited by the availability of basic nutrients and minerals as it requires an adequate supply of many different nutritional factors and nutrient deficiencies in infancy can lead to bone abnormalities. However, the exact mechanism whereby nutrition modulates cellular activity during bone elongation is still not known (Gat-Yablonski et al. 2009).

In most vertebrates, the skeleton represents the main reservoir of Ca, P, and other ions and is in a state of continual exchange with electrolytes found in blood and extracellular fluids. Thus, the skeleton of most vertebrates exerts a massive buffering effect on changes in plasma electrolyte levels and consequently mineral homeostasis (Lall and Lewis-McCrea 2007).

Calcium and phosphorus metabolism

Phosphorus plays a critical role in diverse biological processes, and, therefore, the regulation of phosphorus balance and homeostasis is critical for cell signaling, nucleotide metabolism, energy metabolism, membrane function and bone

mineralization (Sommer et al. 2007). In mammals, phosphorus transport is accomplished mainly by inorganic phosphate transporters in the epithelial cells of the intestine and kidney. Intestinal absorption of Pi is mediated primarily via the type II sodium-phosphate cotransporter (NaPi-IIb) in the brush-border membrane of the intestine, while the reabsorption of Pi in the kidney is mediated by the NaPi-IIa isoform located in the apical membrane of the renal proximal tubule (Werner and Kinne 2001). All of these transporters have been identified in trout and more recently a second isoform of NaPi-IIb as been identified in the pyloric ceaca of trout, which is thought to contribute to the majority of Pi absorption (Sugiura et al. 2000; Sugiura et al. 2003a; Sugiura and Ferraris 2004a; Sugiura and Ferraris 2004b).

Calcium is an essential ion which has a variety of structural and functional roles, ranging from the formation and maintenance of the skeleton to the temporal and spatial regulation of neuronal function and inhibition of the proliferation of cancer cells. Intracellular Ca^{2+}, particularly cytosolic free Ca^{2+}, is an important second messenger and cofactor for proteins and enzymes, regulating key cellular processes such as neurotransmission, motility, hormonal secretion and cellular proliferation. Conversely, extracellular $Ca2^{+}$ is an integral part of the mineral phase of the bone, serves as a cofactor for adhesion molecules, clotting factors and other proteins and regulates neuronal excitability (Khanal and Nemere 2008). The concentration of calcium in extracellular fluid is precisely regulated in all vertebrates and the ability to sense changes in plasma-ionized calcium is mediated by the calcium-sensing receptor (CaSR), which is an integral component in regulating PTH secretion from the parathyroid glands of mammals (Brown et al. 1993). The CaSR is expressed in multiple tissues, including thyroid, kidney, intestine, bone, bone marrow, brain, skin, pancreas, lung and heart (Kos et al. 2003) where the responsiveness to calcium may be determined by variations in expression of CaSR. This receptor has also been identified in the Stannius corpuscles of teleosts where it is thought to have a similar function (Greenwood et al. 2009).

Calcium and phosphate are present in the circulation in both bound and ionized forms. The ionized forms are the most strictly controlled. The importance of both Ca^{2+} and Pi in the body is reflected in the exquisite hormonal regulation associated with each element. Both Ca^{2+} and phosphorus (as "phosphate" or P_i/HPO_4^{-2}) utilize passive diffusion and saturable/facilitated absorption during intestinal uptake (Figure 1.4 A and

B) (Bringhurst and Leder 2006; Goodman 2005; Ramasamy 2006b). When dietary calcium is abundant, the passive paracellular pathway is thought to be prominent. In contrast, when dietary calcium is limited, the vitaminD-dependent transcellular pathway is thought to play a major role. The mechanisms of calcium absorption in the kidney are thought to be very similar to the intestine (Bringhurst and Leder 2006; Ramasamy 2006a).

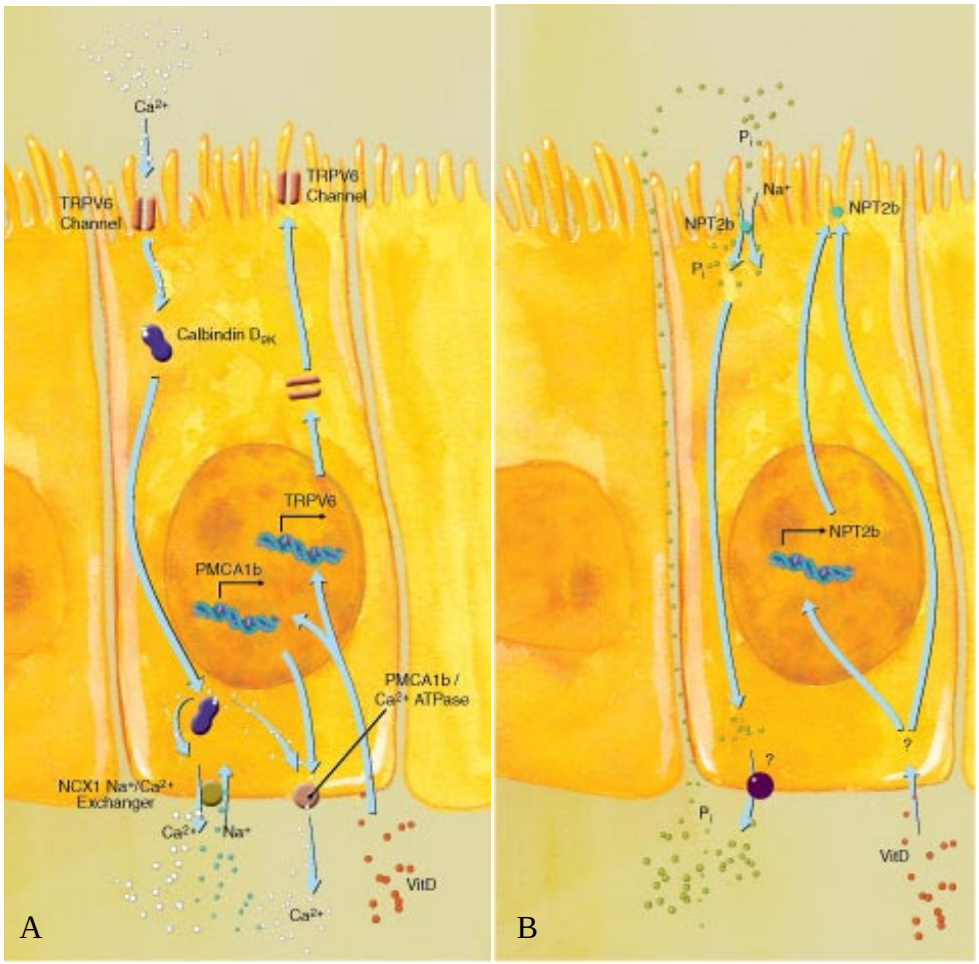

Figure 1.4 - Transcellular A) Ca^{2+} and B) Pi transport in the intestine of tetrapods. Pi transport can also occur by paracellular mechanisms B). Taken from (R&DSystems 2007).

Ca^{2+} enters the cell via an electrochemical gradient and once inside it binds to calbindins Ca^{2+}-binding proteins, transits through the cell and is delivered to one of two basolateral transporters/pumps, a Ca^{2+}-Na^{+} exchanger (NCX1) or plasma membrane Ca^{2+}-dependent ATPase (PMCA1b). The synthesis of calbindins is upregulated by Vit D (Ramasamy 2006b; Bödding and Flockerzi 2004). Paracellular Ca^{2+} and Pi transport is driven by a transepithelial electrochemical gradient (Ramasamy 2006a)

Pi transcellular (or facilitated) transport involves at least three components in the cell; a luminal Na^+/Pi co-transporter, a basolateral Na^+/K^+ ATPase, and an as yet unidentified, but hypothesized, basolateral Pi transporter . The Na^+/Pi co-transporter (or NaPiIIb) is an 80 kDa, 8-transmembrane domain protein that simultaneously transports one Na^+ and one P_i ion into the cell. Once internalized, Pi exits the cell on the basolateral side. NaPiIIb is reported to be positively regulated by VitD is also likely to respond to differences in intracellular Na^+ concentration. Some passive paracellular Pi transport may occur, although the transcellular mechanism is likely to be primary mean transport (Goodman 2005; Ramasamy 2006b).

Regulation of Ca^{2+} and Pi metabolism

In tetrapods, the main endocrine factors controlling Ca^{2+} and ionic phosphate (Pi) are parathyroid hormone (PTH), calcitonin (CT), and 1,25-dihydroxyvitamin D (Vit D). PTH is synthesized by cells of the parathyroid glands, it acts as a hypercalcemic factor and its shows opposite effects to CT which is a hypocalceamic hormone produced in parafollicular cells of the thyroid gland in amphibians, birds and mammals. PTH accomplishes its hypercalceamic effect in three ways; i) it induces the differentiation and activation of osteoclasts which mobilize Ca^{2+} and Pi from bone; ii) it stimulates the production of 1,25-dihydroxyvitamin D, which by turn enhances the absorption of Ca^{2+} from the small intestine and iii) PTH also stimulates tubular reabsorption of Ca^{2+} in the kidney (Mundy 1991; Mundy and Guise 1999; Guerreiro et al. 2007).

Fish, whether living in seawater or freshwater, are surrounded by a readily available source of ionic Ca^{2+}, so the need for a hypercalcemic factor was considered unnecessary. This idea persisted until the 1980's and was reinforced by the identification in fish of an endocrine factor with anti-hypercalcemic action, stanniocalcin (STC) (Bonga and Pang 1991; Guerreiro et al. 2007). However, in the 1980's and 90's evidence accumulated for the existence of a hypercalcemic factor and culminated in the isolation of PTHrP in 2000 (Flanagan et al., 2000; Power et al., 2000). The action of the PTH-like factors in fish is not completely clear, they are involved in many physiological processes not necessarily related to Ca^{2+} regulation, but studies indicate that PTHrP may well be the equivalent to PTH in tetrapods acting as a hypercalceamic factor on branchial, intestinal, and renal mechanisms (Canario et al.,

2006). Other endocrine factors, such as cortisol and estradiol have also been shown to increase Ca^{2+} uptake, and therefore it seems that hypercalcemic factors are necessary in fish at least in periods of high Ca^{2+} demand (Flik and Perry 1989; Guerreiro et al. 2002; Guerreiro et al. 2007). The effects of calcitonin in fish are controversial since they depend on the fish species and experimental conditions used (Shinozaki and Mugia 2002) although it is known to act as an hypocalcemic hormone by inhibiting Ca uptake by the gill.

Regulation of Pi homeostasis has long been thought to be under the control of calcium-regulating hormones PTH, CT and Vit D. However, recent advances have shed new light on the mechanisms that control phosphate balance in normal and pathological states and suggest that the previous view was not inetrely correct (Berndt and Kumar 2008; Kiela et al. 2009). In the past decade phosphatonins have been identified and are important in P regulation. These molecules, FGF-23, sFRP-4, MEPE were first described as having circulating phosphaturic activity and were present at elevated levels in diseases characterized by: imbalance in phosphorus, abnormal 1,25-dihydroxyvitamin D3 levels and defects in skeletal mineralization. These diseases include oncogenic osteomalacia (OOM), autosomal dominant hypophosphatemic rickets (ADHR), and X-linked hypophosphatemic rickets (XLHR) (Schiavia and Moe 2002). It was the study of these diseases and their relation with phosphorus that triggered a series of studies about the homeostasis of phosphorus and clarified fundamental aspects of Pi metabolism until recently unknown. Although there is no question as to the importance of these factors in the pathophysiology of phosphate in the various disorders described, it still remains somewhat uncertain as to whether phosphatonins play a role in normal phosphate homeostasis (Berndt and Kumar 2008).

A recent theory is the existence of “phosphate sensors” (Suzuki et al. 2004; Lamarche et al. 2008) identified in unicellular organisms and individual cells of the intestine and kidney which can sense changes in extracellular (or in some cases intracellular) Pi concentrations. The sensors, alter intracellular protein metabolism, generally by altering the phosphorylation state of intracellular proteins, and subsequent nuclear transcription events. Proteins synthesized in response to changes in gene transcription increase the efficiency with which phosphorus is retained by the cell and may be components of the yet unknown cellular Pi sensor (Berndt and Kumar 2008). These are short-term and

rapid adaptive responses that occur independent of hormones previously thought to be important in phosphorus homeostasis, and may play a larger role than previously appreciated in the regulation of phosphorus homeostasis. Several hormones and regulatory factors such as the vitamin D endocrine system, parathyroid hormone, and the phosphatonins (FGF-23, sFRP-4, MEPE) among others, seem to play a role only in the long-term regulation of phosphorus homeostasis (Berndt and Kumar 2008).

In fish there is a large variety of factors that regulate P metabolism this was demonstrated *in vitro* in flounder renal tubule cell where net Pi reabsorption was increased by salmon stanniocalcin, rat prolactin, salmon/flounder somatolactin, bovine PTH, and salmon growth hormone (Lu et al. 1994; Lu et al. 1995) and phosphatonins are also expressed in fish (Bianchetti et al. 2002). Plasma Pi homeostasis depends on balanced rates of Pi uptake by the small intestine, reabsorption or excretion by the kidneys, and resorption or deposition into bone. In fish, chronic phosphorus deficiency is associated with decreases in growth, feed efficiency, appetite and bone strength, as well as increases in body fat and the incidence of bone deformity (Kirchner et al. 2007). Calcitonin is another hormone that affects P metabolism in mammals but its role in P balance in fish is not clear although it does inhibit Ca uptake by the gill (Wagner et al. 1997).

Fish as models for biomedical research

Fish have been adopted over the last decade as models of interest for biomedical research. The biodiversity of fish and their success (most abundant extant vertebrates) makes them of interest from a number of perspectives, not least their adaptability to diverse environments and the associated innovations from a structural to molecular perspective. In the particular case of bone metabolism/formation and mineral balance numerous genes and their protein products implicated in these processes are common in fish and tetrapods and highlight an area of potential interest. Model teleosts allow manipulation of suspected etiological correlates such as the one between hormones and mineral levels. Therefore analysis of the underlying molecular mechanism in fish will help to identify novel genes and regulatory pathways of bone homeostasis and skeletal disorders applicable also in higher vertebrates (Renn et al. 2006).

The model teleost used was the rainbow trout for which numerous studies of nutrition and in particular dietary P requirements and digestibility exist and for which candidate genes responsive to P restriction have been identified (Sugiura et al. 2000; Sugiura et al. 2003b, 2003a; Sugiura et al. 2004b).

1.2 Objectives

The aim of the present work was to establish how dietary levels of P and Ca influenced skeletal ontogeny and also associated biochemical, molecular and endocrine parameters in teleost fish. The objectives of the thesis were:

1) Determine the effect of dietary P and Ca^{2+} in the body composition of developing rainbow trout as early as first feeding.
2) Access the effect of dietary P and Ca^{2+} on skeletal ontogeny and determine the density of bone at juvenile stage by Xray.
3) Relate the appearance of malformations with the dietary conditions.
4) Characterize the morphology of calciotropic glands, Ultimobranchial Gland and corpuscles of Stannius.
5) Evaluate the expression of genes known to be modified in adult trout and relate these findings with the expression of genes involved in bone formation and development.
6) Determine the expression of mineral regulatory endocrine factors, stanniocalcin and calcitonin.

PART 2

GENERAL MATERIALS AND METHODS

2.1 Materials and Methods

The methods used to perform the experiments described in this thesis, are resumed in Figure 2.1.

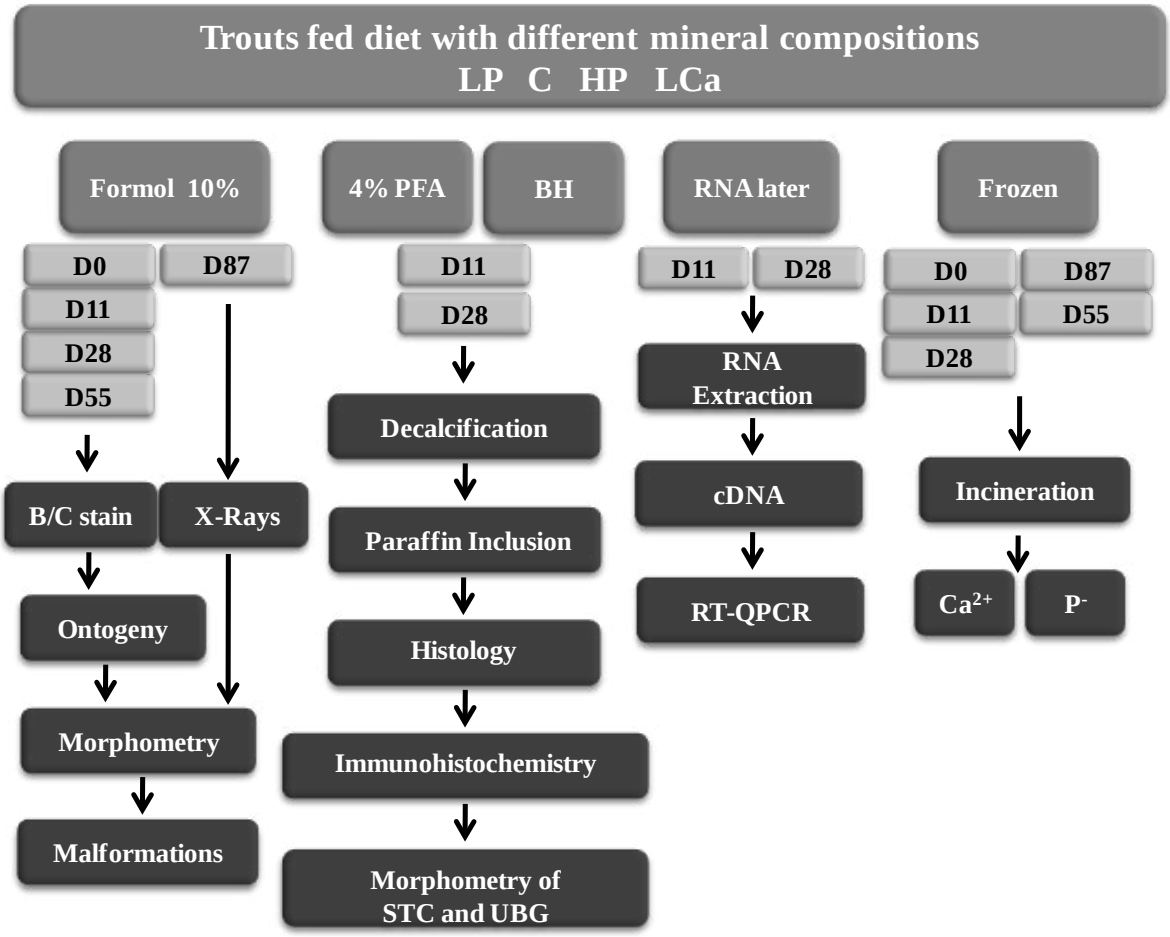

Figure 2.1 - Flow chart describing the main methodologies used for analysis of the experimental material collected. The experiment consisted in feeding trouts (*Oncorhynchus mykiss*) aged 51dpf diets with different contents of phosphorus and calcium (low phosphorus - LP; compound - C; high phosphorus - HP; LCa - low calcium). The experiment occurred for 87 days after first feeding. Trouts were analyzed at 5 sample points during development (D0, D11, D28, D55 and D87), and depending in the type of analyses required, preserved in formol 10%, 4% PFA, BH, RNAlater or just frozen. To study skeletal ontogeny, morphometry and occurrence of malformations samples were preserved in formol 10%, used for B/C (bone and cartilage) staining and X-ray. To determine body composition in minerals samples were preserved frozen. The study of glands producing hormones involved in mineral metabolism was performed in samples fixed in Bouin-Hollande and PFA followed by histology and immunohistochemistry and to determine the effect of diet on gene expression samples were preserved in RNAlater until analysis by RT-QPCR .

2.1.1 Experimental fish and dietary trial conditions

All-female diploid rainbow trout (*Oncorhynchus mykiss*) embryos (230 degree-days post-fertilization) were provided by Aqualande (Landes, France) to the INRA experimental fish farm in Lées-Athas (Pyrénées-Atlantiques, France). One day before the swim-up stage, fry were transferred to the INRA experimental fish farm in Donzacq (Landes, France) and randomly distributed into 18 tanks (600 larvae per 50 l fiberglass tank) supplied by spring water at 17 ± 1°C. From the swim-up stage (53 days, 450 degree-days post fertilization) defined as day 0 of the experiment, fish (initial mean weight: 0.10 ± 0.01 g) were hand-fed six times per day to excess or visual satiety. Each diet was distributed to 3 replicate tanks over a 12-week growth trial.

2.1.2 Experimental diets

Four semi-purified casein based diets were formulated (Table 2.1). Diets were isoproteic (52% dry matter) and isolipidic (16% dry matter) and contained graded levels of phosphorus (P) and calcium (Ca). The basal diet LP contained only P supplied by casein, 0.45%. Other diets C and HP were supplemented with 0.8 and 1.6% available P supplied as a 1:1 mixture of NaH_2PO_4/KH_2PO_4 resulting in 1.2 and 2.2% P, respectively. These four diets were supplemented with 1% Ca supplied as $CaCo_3$ whereas another diet LCa, supplemented with 0.8% available P, was Ca-free.

Table 2.1 - Formulation and composition of experimental diets (g/100g dry weight)

Diet	LP	C	HP	LCa
Ingredients				
Casein-dextrin basis[a]	82.5	82.5	82.5	82.5
Vitamin mixture[b]	5	5	5	5
Mineral mixture without P and Ca[c]	2.5	2.5	2.5	2.5
NaH_2PO_4/KH_2PO_4 (50/50)	0.00	3.36	6.72	3.36
$CaCO_3$	2.50	2.50	2.50	0.00
α-Cellulose	7.50	4.14	0.78	6.64

Available P	0.0	0.8	1.6	0.8
Available Ca	1.0	1.0	1.0	0.0
Proximate composition				
Dry matter (%)	93.4	92.6	94.0	93.6
Crude protein	51.3	51.1	52.7	52.4
Total lipid	16.3	16.8	16.7	16.9
Gross energy (kJ/g dry matter)	23.0	22.5	21.9	23.1
Ash	4.7	7.3	10.6	6.1
Total phosphorus	0.5	1.2	2.2	1.3
Total calcium	0.7	0.8	0.8	0.1

[a]Casein-dextrin basis (% diet): 52% casein (VWR Prolabo 22 544.292, Fontenay-sous-Bois, France); 0.65% D,L-methionine (Ajinomoto Eurolysine, Paris, France); 0.85% L-arginine HCl (Ajinomoto Eurolysine); 8% soybean lecithin (Louis François, St Maur des Fossés, France); 8% fish oil (La Lorientaise, Lorient, France); 12% dextrin (Sigma D2256, Saint-Quentin Fallavier, France); 1% sodium alginate. [b]Vitamin mixture (per kg vitamin mix): retinyl acetate, 500 000 IU; cholecalciferol, 250,000 IU; DL-α-tocopherol acetate, 5 g; menadione, 1 g; thiamin-HCl, 0.1 g; riboflavin, 0.4 g; D-calcium panthothenate, 2 g; pyridoxine-HCl, 0.3 g; cyanocobalamin, 1 mg; niacin, 1 g; choline, 100 g; ascorbic acid (L-ascorbyl-2-polyphosphate), 5 g; folic acid, 0.1 g; D-biotin, 20 mg; meso-inositol, 30 g. All ingredients were diluted with α-cellulose. [c]Mineral mixture (g/kg mineral mix): KCl, 180; KI, 0.08; NaCl, 80; $CuSO_4 \cdot 5H_2O$, 6; $ZnSO_4 \cdot 7H_2O$, 8; $CoSO_4$, 0.04; $FeSO_4 \cdot 7H_2O$, 40; $MnSO_4 \cdot H_2O$, 6; MgOH, 248; Na_2SeO_3, 0.06; NaF, 2. All ingredients were diluted with α-cellulose.

2.1.3 Sample collection

To access the effect of the experimental treatments at different developmental stages, samples of trout fry were withdrawn on day 0 (first feeding) and from each tank on days 11, 28, 55 and 87. Food as withheld for 24 h prior to sampling. The stage of development of the fish correspondent to each sample point is described in Table 2.2.

Table 2.2 - Stage of development at each sample point where (dpf are days post fertilization and dpff are days post first feeding).

Stages		
dpf	**dpff**	**Main events**
51	**D0**	Inflation of gas bladder and beginning of exogenous feeding
62	**D11**	Yolk sac resorption
79	**D28**	1 month after first feeding
106	**D55**	2 months after first feeding
138	**D87**	3 months after first feeding

The fish were anaesthetized by immersion in diluted 2-phenoxyethanol (1%v/v) and sampled for wet weight determination, fixed in 10% formalin, 4% paraformaldehyde or Bouin-Hollande for histological and morphological analysis; fixed in RNAlater (Sigma-Aldrich, France) and stored at -20ºC for molecular biology or chemical analysis.

2.1.4 Morphometry

Samples from each sample point and for all groups were fixed in formol 10% (Merck, VWR, Portugal) and stored at 4ºC until analysis.

2.1.5 Endocrine gland with calciotropic action

Samples from D11 and D28 were used for immunohistochemistry and histology. They were fixed in Bouin-Hollande fixative (Annex 1) for 5-7 days, at room temperature, followed by washes in tap water over 2 hours and distilled water for 30 minutes, and storage in ethanol 70% at 4ºC.

Other batches of samples were also fixed in 4% paraformaldehyde (Annex 1) overnight at 4ºC. The fixed samples were washed 3 x 15 minutes with sterile PBS pH7.4 (Annex 1) followed by 15 minutes in sterile water, samples were stored in 70% ethanol and stored at -20ºC.

Fixed samples where then decalcified in EDTA and processed for paraffin embedding (Annex 1), followed by sectioning at 5 μm in a rotating vibratome (Leica RT2125) and mounting of sections on APES (Annex 1) treated slides.

2.1.6 Gene expression

For gene expression analysis samples where immersed in 10 volumes of RNAlater (Sigma-Aldrich, Madrid, Spain) fixed overnight at 4ºC and stored at -20ºC, until analysis. Five individuals per experimental group (40 samples in total) where used for this analysis.

PART 3

EFFECTS OF DIETARY PHOSPHORUS AND CALCIUM LEVEL ON GROWTH AND SKELETAL DEVELOPMENT IN RAINBOW TROUT (*Oncorhynchus mykiss)* FRY

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3.1 Introduction

Calcium (Ca) and phosphorus (P) are closely related to the development and maintenance of the skeletal system and its stability is maintained by a solid phase of calcium phosphate (Lall and Lewis-McCrea 2007). Studies with monogastric animals have shown that an optimum Ca to P ratio is important and increasing the Ca to P ratio of the diet interferes with the absorption of P and, conversely, a high P to Ca ratio may restrict Ca absorption (Calvo 1993; Calvo et al. 1996). The exclusive source of calcium in tetrapods is the diet but fish can also obtain calcium from the surrounding water [freshwater 10–150 mg/l Ca and seawater 400 mg/l Ca] through the gills, fins and oral epithelial tissue. This means dietary Ca deficiency or Ca-P imbalance observed in tetrapods rarely becomes a problem in fish. In common with tetrapods calcium is deposited in the bone of fish but it is also deposited in scale and skin (Skonberg et al. 1997).

In tetrapods and fish the only source of P is the diet and in fish its regulation is considered more critical than that of Ca because fish must effectively absorb, store, mobilize, and conserve phosphate in both FW and SW environments. The absorbed P in teleosts accumulates in soft and skeletal tissue (heart, liver, kidney, muscle and blood, etc.) and is essential for growth and metabolism (Lall and Lewis-McCrea 2007). When dietary intake is insufficient fish draw upon their tissue reserves to supply phosphorus for metabolic and growth needs but the amount that can be supplied from tissue reserves is limited, and at this point clinical signs of phosphorus deficiency may start to arise, and include poor growth, reduced feed efficiency and skeletal deformities, poor bone mineralization, low ash and high lipid content in the whole body (Ye et al. 2006).

In humans in the intake of Pi has increased steadily as consumption of Pi-rich foods increased by approximately 17% in the decade leading up to 1993. These surveys also suggested that the use of Pi as a food additive may continue to increase, so that excess intake may become an issue (Calvo 1993; Calvo et al. 1996; Jin et al. 2006; Jin et al. 2007).

The main causes of Pi deficiency in humans are genetic disorders which involve impairment of kidney function or even malnutrition (Lotz et al. 1968; Sommer et al. 2007; Knochel 2000). The involvement of Pi in a range of disorders in mammals has resulted in a number of studies in vertebrates which indicate that Pi provokes up- and down-regulation of several pivotal genes (Brown et al. 1993; Dougall et al. 1996; Skonberg et al. 1997; Baeverfjord et al. 1998; Velasco et al. 1998; Chavez-Sanchez and Ross 2000; Sugiura et al. 2000; Sugiura and Ferraris 2004a; Sugiura et al. 2004b; Ye et al. 2006; Uyan et al. 2007). However, much more work is required to understand the effects of dietary mineral metabolism on homeostasis and development of the vertebrate skeleton.

Bone and cartilage may develop during embryonic, juvenile or adult stages under normal ontogeny as well as during pathological states, wound repair and bone regeneration. The knowledge of gene action, cell differentiation, morphogenesis, species differences in phenotypic expression of genetic abnormalities and rates of deposition of calcium and phosphorus and other trace elements in vertebrate cartilage and bone are essential to understand both normal and pathological states of the skeletal system (Lall and Lewis-McCrea 2007). Teleost fish are proving to be a useful model to fill-in some of the gaps in human vertebral research namely idiopatic scoliosis. Defects of the vertebral system in both fish and humans are a consequence of genetics and physiology and may be associated with mineral homeostasis, developmental problems (eg. fused vertebrae) and infections caused by viruses or parasites. In fact, the most common type of deformities seen in fish are associated with the vertebrae, and most of these occur during development (Brown and Nunez 1998); (Aubin et al. 2005; Gorman and Breden 2007). For example, recently, a mutant of zebrafish was described that has disturbed bone growth, uneven mineralisation, and apparent bone weakness. The phenotype was caused by mutations in *col1a1* and resembled those found in human osteogenesis imperfecta (Fisher and DeGeorge 1967; Renn et al. 2006).

The objective of the present study was to determine the effect of different levels of dietary phosphorus and calcium on growth, skeletal development, body composition and morphology of rainbow trout fry from first feeding.

3.2 Methods

Skeletal ontogeny and morphology

3.2.1 Chemical analyses

With the objective of determining the effect of experimental diet on the mineral content of fry the proximate composition of fry was determined on groups of whole fish. For this purpose individuals from all experimental sample points and diets (see chapter 2) were used, and the analysis performed in triplicate (one for each tank) except for D55 high phosphorous (HP) that only had 2 experimental tanks and D87 HP that only had 1 experimental tank due to high mortality in this group.

3.2.2 Dry matter

Fresh samples were weighed to determine the wet weight and dried at 105ºC for 24h or until samples had a constant weight. At this point the samples were weighed again to determine dry weight and dry matter was calculated as a % of dry weight/ wet weight.

3.2.3 Ash content

Frozen individual fish were weighed and then dried to a constant weight (65°C for 3 days), this weight represents the dry weight. The samples were then ground-up, placed in crucibles and placed in a furnace (500°C for 24 h) to ash. The ash content of each individual was established as a % of ash weight/wet weight.

3.2.4 Determination of Ca and P

2.5 mg of ash corresponding to each experimental group were placed in tubes of 1.5 ml and digested with 150 µl nitric acid for 24 h at 25°C. The resulting digest was neutralized with equal volumes of 2 M NaOH. Total calcium in the digests was measured in duplicate using a commercial colorimetric assay Calcium-oC v/v,

(Spinreact, Spain). This technique is based on the formation of a coloured complex between the calcium in the sample and *o*-cresolftalein in alkaline medium, the color intensity of the complex obtained which is directly proportional to the quantity of calcium present in the sample was measured in a plate reader Benchmark (Biorad) device with a 570nm filter. The results were expressed as % calcium/wet weight

Total phosphorus in the digests was measured in duplicate using a commercial colorimetric method Phosphorus-UV (Spinreact, Spain). This technique is a direct method for the determination of inorganic phosphorus, this reacts with ammonium molybdate creating a phosphomolybdic complex of yellow color. The absorbance of the complex is proportional to the concentration of inorganic phosphorus in the sample and was measured in a plate reader Benchmark (Biorad) using a 340 nm filter. The results were expressed as % phosphorus/wet weight.

3.2.5 Whole mount skeletal preparation and analysis

In order to compare skeletal development and the effect of the experimental diets, 5 individuals from D0 and 15 individuals from D11 and D28 from each dietary group, fixed in 10% formalin, were stained for cartilage and bone with Alcian blue and Alizarin red S according to (Faustino and Power, 1998). Cartilage and bone development was studied in whole animals and the staining procedure was done simultaneously in all samples to avoid variability. Prior to staining the standard length (SL) of each sample was measured from the tip of the snout to the base of the caudal fin complex. Rainbow trout samples were washed several times in H_2Od, in order to remove the fixative, followed by staining for up to 1 hour in a 2% Alcian blue 8GX (Sigma Aldrich, Madrid) solution (Annex 1). The samples were depigmented in 3%H_2O_2 in 0.5% KOH to permit better visualization of stained structures, the length of treatment (10 to 45 minutes) varied with the size of the fish. Maceration of specimens in a solution of 1% KOH for 1 hour caused the muscle to become transparent and the skeleton was then stained in a 0.25% solution of Alizarin red S (Sigma Aldrich, Madrid) in 0.5% KOH overnight. To preserve samples they were placed in solutions with increasing concentrations of glycerol/0,5% KOH (1:3, 1:1, 3:1) and were stored in 100% glycerol and the analysis of cartilage and bone development subsequently carried out with the aid of a stereomicroscope (Olympus SZ-40).

In order to compare the skeletal development of the fish from different treatment groups, a number of dermal and endochondral structures from the axial skeleton and fins were selected for observation in the stained samples (Table 3.1).

Table 3.1 - Skeletal structures studied to assess the progression of chondrogenesis and osteogenesis in rainbow trout fed diets supplemented with different levels of available P (0, 0.8 and 1.6%) and Ca (0 and 1%)

Vertebral Column	Pleural Ribs Vertebrae Centra Neural Arches Hemal Arches	
Pectoral Fin	Cleitrum Coracoid Scapula Radial	Postcleitrum Supracleitrum Actinosts Rays
Dorsal Fin	Pterygiophores Rays	
Pelvic Fin	Basipterigium Rays	
Anal Fin	Pterygiophores Rays	
Caudal Fin	Hypurals Epurals Rays Urostyle	

The initiation and completion of chondrogenesis and the initiation and degree of ossification (intensity of Alizarin red staining) were analyzed. The structures were classified by developmental stage of the skeletal as being: i) cartilage endochondral, when the structure was heavily stained blue; ii) transition endochondral, was one of two situations, when the structure had poor affinity for alcian blue and became transparent or when it already started to calcify and had some affinity for alizarin red S; iii) ossified endochondral, in this case the endochondral structure was fully stained red, but still the intensity can vary; transition dermal structures that are not visible by this method until the beginning of calcification gain a pink coloration; ossified dermal, when the structure is fully stained red, again the intensity of the stain varies with the amount of calcium present).

3.2.6 Morphometric analysis

Whole mount staining of cartilage and bone is a quick and useful method for visualization of the skeleton of small specimens; however for larger specimens X-ray is more useful. After preliminary studies the optimal exposure mode was established for X-ray studies of rainbow trout fixed in formalin (10%) and sampled on D87 of the experiment (n = 9 for diet LP, C, LCa and n = 6 for diet HP). Whole rainbow trout fixed in 10% formalin were washed in dH_2O several times to remove the fixative. They were analyzed using a Senographe DMR Mammography System (General Electric Medical Systems), on the manual exposure mode, to give 40 mAs and 40 kV. Several rainbow trout of the same age and approximate size were X-rayed simultaneously. All X-rays were carried out on rainbow trout samples using exactly the same conditions. In order to read and process the digital X-rays a FUJIFILM Computed Radiography CR Console connected to the Mammography Workstation MV-SR657 FCR PROFECT CS was used, and the digital X-rays archived as JPEG 8bit images until subsequent analysis. This analysis was performed in the radiology department of the Hospital Distrital de Faro. The digital radiographies of rainbow trout were analyzed using Image J (freeware – NIH, http:\\www.rsbweb.nih.gov.ij). The morphometric measurements performed in each of the X-rayed sample were: standard length (SL), height and length of vertebrae and the interneural arch space of the vertebral column (figure 3.1). Based on measurements of height (H) and length (L) the vertebral area was also established and compared between different experimental groups of rainbow trout by assuming that vertebrae approximately resemble a cylinder and applying the formula, $Area_{veterbra}=2\pi$ H/2 L.

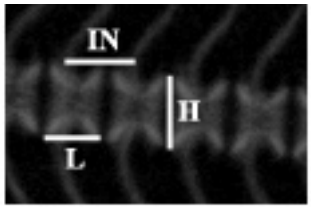

Figure 3.1 – X-ray image from a section of the vertebral column of D87 trout, indicating the measurements taken; H – height, L – length and IN – interneural arch space length.

In order to simplify the measurements in each individual 3 vertebrae in the midpoint of the four different anatomical regions previously described in the rainbow trout vertebral column (Fig. 3.2, see Kacem et al., 2003) were analyzed.

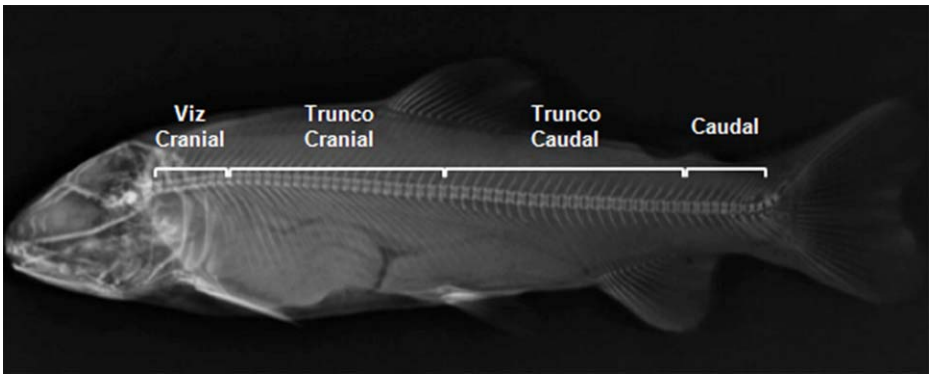

Figure 3.2 - Radiography of rainbow trout showing the four distinct regions considered during measurement of the vertebral column: Vizcranial (V1-V8); Truncocranial (V9-V30); Truncocaudal (V31-V51) and the Caudal region (V52-V64).

3.2.7 Ossification level at D87

The degree of ossification of X-rayed rainbow trout was estimated using "the mean grey value approach" and image analysis software, Image J (NIH). Five vertebrae situated in the midpoint of each of the anatomical areas used for morphometry were selected for measurement in rainbow trout from each experimental group (n = 9 for diet LP, C, LCa and n = 6 for diet HP). The analysis is based on the fact that bone which is more calcified appears whiter in X-rays and has a higher mean grey value (Versluis et al., 2000).

3.2.8 Malformations analyses

The determination of the incidence of malformation in the experimental groups was registered first by external observation of the animals to detect malformations that compromised the external appearance; followed by detailed analysis of cartilage/bone in whole mount stained individuals from sampling points D11, D28 and D55 using a stereomicroscope (Olympus SZ-40) and digital X-ray images for D87 individuals using Image J (NIH) software. At D11 and D28 the number of individuals analyzed in each

group was 15, at D55, 5 individuals were analyzed and at D87 9 individuals were analysed for LP, C, LCa groups and 6 for the HP group. The same structures used to determine skeletal ontogeny were analyzed (Table 3.1). For simplicity malformations were grouped into the main categories according to the structure affected or specific type of abnormality, the categories also varied according to the sampling time point. Several parameters were calculated: % malformation incidence (total number of malformations in the group/number of individuals in the group) external or internal; % incidence of malformations within experimental groups, in which the frequency of each abnormality is considered as a proportion of the total number of abnormalities (100%) detected per experimental group; Incidence of malformations category observed in trout in all experimental groups at D87 (type of malformation incidence/total number of malformations in all groups).

3.2.9 Statistical analyses

All results are given as means ± standard deviation (SD). Differences between dietary groups were analyzed using one-way ANOVA. The Newman–Keuls multiple range test was used to compare means when a significant difference was found. Weight and morphometric measurement data were log-transformed before analysis, and differences were considered significant when $P < 0.05$. All the statistical analyses were performed with SigmaStat 3 (SPSS, Chicago, IL, USA)

3.3 Results

3.3.1 Fry performance

Fry readily accepted the experimental diets from the beginning of the experiment and maintained normal behaviour throughout the experimental period. As fry were fed to excess or visual satiety, feed intake and feed efficiency could not be calculated but these parameters were evaluated to check that no group was food-restricted.

Weight and Standard length

Fish fed with different experimental diets did not present significant differences in weight or standard length over the duration of the experiment until day 87 post first

Table 3.2 - Growth performance and survival of rainbow trout fry fed diets supplemented with different levels of available P (0, 0.8 and 1.6%) and Ca (0 and 1%).

Diets	**LP**	**C**	**HP**	**LCa**
Survival (%)				
Day 11	91 ± 2	94 ± 1	93 ± 3	94 ± 1
Day 28	78 ± 7[a]	66 ± 13[a]	42 ± 9[b]	76 ± 5[a]
Day 55	73 ± 3[a]	64 ± 13[a]	24 ± 21[b]	74 ± 6[a]
Day 87	72 ± 4[a]	63 ± 13[a]	10 ± 17[b]	74 ± 6[a]
Mean wet weight (g)				
Day 11	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
Day 28	0.55 ± 0.05	0.54 ± 0.03	0.41 ± 0.04	0.50 ± 0.05
Day 55	1.2 ± 0.1	1.3 ± 0.2	1.1 ± 0.2	1.0 ± 0.1
Day 87	3.7 ± 0.9	4.2 ± 1.4	4.1	2.7 ± 0.1
Standard Length (mm)				
Day 0	19.7 ± 2.1	19.7 ± 2.1	19.7 ± 2.1	19.7 ± 2.1
Day 11	21.2 ±1.2	22.1 ± 1.2	22.2 ± 1.2	22.3 ± 0.9
Day 28	29.0 ± 2.3	30.2 ± 2.8	28.3 ± 2.2	30.2 ± 1.4
Day 55	41.6 ± 4.0	42.3 ± 4.7	41.6 ± 5.4	39.7 ± 3.3
Day 87	59.4 ± 7.4	59.1 ± 6.9	62.7 ± 9.5	52.0 ± 9.1

Values are means ± SD except for data sharing an asterisk. Within rows, means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by a Newman-Keuls test.

feeding (Table 3.2), indicating that in early stages of trout, modifications in the mineral content of the diet does not significantly affect growth in length. In the last sample point, group LCa seemed to have a decreased growth rate, the trend for reduced growth was already evident from day 55, but individual variation within the group meant it was not statistically different between dietary treatments.

Survival

At the end of the experiment the survival of fish fed with diets LP and LCa was similar to those fed with the compound diet, about 65% (Table 3.2). In contrast, fish that were fed with excess phosphorus had high mortality rates, at D28 only 42% of the individuals survived, while at D55 the survival rate was of 24% and at D87 only 10% of the initial fish were still alive.

3.3.2 Mineral content

Dry matter and Ash content

The dry matter content of the fry increased during larval development (Table 3.3) and was independent of fry size, no significant effect of dietary P or Ca supplementation was noticed on dry matter content. In terms of ash content, from day 28 onwards group LP displayed a lower whole body ash content compared to the other groups this difference persisted until the end of the experiment. The LCa group showed no difference in ash content relative to the compound diet group.

Calcium and Phosphorus content

As can be seen on Table 3.3, the body content in calcium increased with age as expected, at D11 the content was similar in all groups, ranging from 0.62% in LP to 0.74% in C group and levels increased from there onwards. At D28 Ca reached 0.97% in group LP, C and LCa were composed of 1.9% calcium and HP group had the highest amount, about 2.1%. At D55 the amount of calcium had approximately doubled in all the groups. At day 87 groups C and LCa had a similar calcium content 4.3 and 4.5% respectively while group HP had 6% and group LP continued to have the lowest value, 3.4% calcium/wet weight. There were no statistically significant differences between treatments.

Table 3.3 - Whole body composition of rainbow trout fry fed diets supplemented with different levels of available P (0, 0.8 and 1.2) and Ca (0 and 1%)

Diets	**LP**	**C**	**HP**	**LCa**
Dry matter (%)				
Day 11	14.2 ± 0.5	14.2 ± 0.4	14.3 ± 0.2	14.2±0.2
Day 28	16.4 ± 0.3	17.3 ± 0.6	16.5 ± 0.3	17.0±0.5
Day 55	18.2 ± 0.8	18.1 ± 0.3	18.0 ± 0.0*	17.9±0.5
Day 87	20.5 ± 0.3	21.1 ± 0.8	20.8**	20.7±0.2
Crude ash (% wet weight)				
Day 11	1.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.2	1.1±0.1
Day 28	1.3 ± 0.0^{a}	1.8 ± 0.0^{b}	1.8 ± 0.0^{b}	1.7±0.1^{a}
Day 55	1.8 ± 0.1^{a}	2.3 ± 0.1^{b}	2.2 ± 0.0^{*b}	2.3±0.1^{b}
Day 87	2.0 ± 0.1^{a}	2.5 ± 0.1^{b}	2.4^{**b}	2.6±0.1^{b}
Total calcium (% wet weight)				
Day 11	0.62 ± 0.05	0.74 ± 0.05	0.67 ± 0.03	0.69±0.12
Day 28	0.97 ± 0.28	1.89 ± 0.06	2.07 ± 0.25	1.87 ± 0.27
Day 55	2.04	3.05	3.29 ± 0.38	3.01
Day 87	3.40 ±1.62	4.26 ± 0.07	5.99**	4.45±0.3
Total phosphorus (% wet weight)				
Day 11	0.21 ± 0.01^{a}	0.25 ± 0.00^{b}	0.26 ± 0.00^{c}	0.24±0.01^{b}
Day 28	0.22 ± 0.01^{a}	0.32 ± 0.01^{b}	0.33 ± 0.00^{b}	0.32±0.01^{b}
Day 55	0.31 ± 0.01^{a}	0.40 ± 0.00^{b}	0.39 ± 0.00^{*b}	0.41±0.03^{b}
Day 87	0.35 ± 0.00^{a}	0.46 ± 0.01^{b}	0.43 **	0.47±0.02^{b}

Values are means of 3 rearing tanks ± SD except for data sharing an asterisk (*), only two rearing tanks due to the total mortality in the other tank and data sharing two asterisks (**), only one rearing tank due to the total mortality in the two other tanks. Within rows, means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by a Newman-Keuls test. (no superscript, only 1 individual no statistic analysis performed(***).

When the increase in calcium between sample points was determined (Figure 3.3) it is very clear that for groups C and LCa the rate of calcium accumulation, 1.2%, is similar and constant for all the sample points. Group LP which had the lowest calcium content at all sampling points seemed to increase the accumulation from 0.35% between D11 and D18 to 1.06% between D28 and 55 which is very similar to group C. For samples of the HP group the increase in Ca content is similar to the control but from D55 to D87 there is a tremendous increase in calcium content.

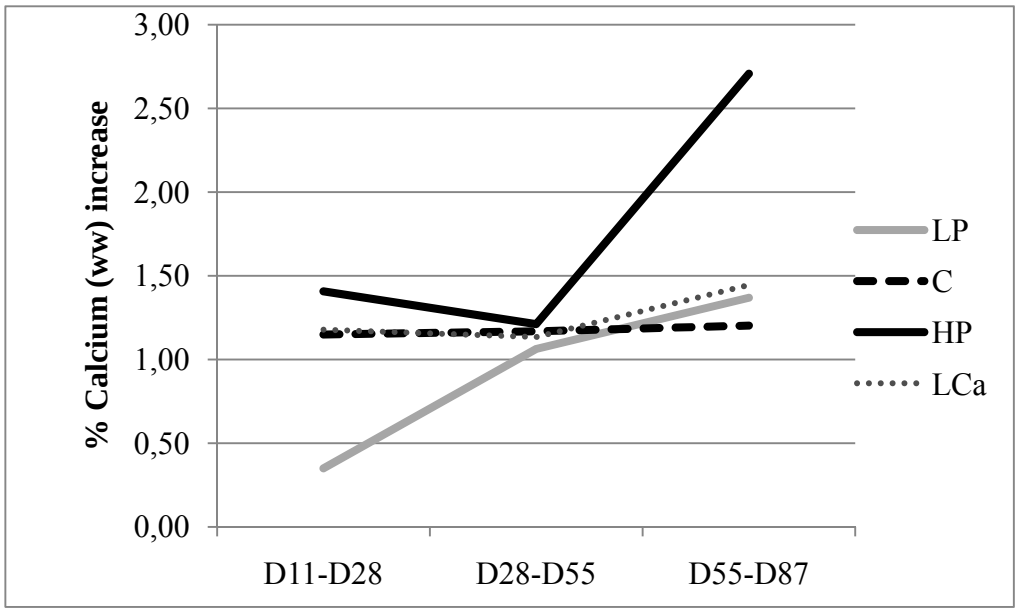

Figure 3.3 - Gain of calcium (% wet weight) between sampling points.

Similar to what happens with the whole body calcium content, the P content increases with age but with a smaller proportion rise, at D11 it varies from 0.24% in LCa, 0.21% in LP, 0.26% in HP and 0.25% in C. At D28 the phosphorous content of LP samples where similar to D11 0.22% and all the other groups, C, LP and LCa, increased whole body P content to about 0.32%. From there onwards the P content of trout fry increased with LP always having a lower P content relative to the other groups. At the end of the experiment groups C and LCa had the highest P content 0.46%, HP group 0.43% and LP only 0.35%.

In Figure 3.4 the pattern of phosphorus accumulation in different groups during the experiment is illustrated. Group LP, from D11 to D28 had the lowest increase with only 0.01%, but from D28 to D55 there is a big increase of 0.08% which leaves it at the level of C and LCa groups, which have a similar assimilation rate. The HP group seems to have the highest incorporation of phosphorus between D11 and D28, 0.08% and from then onwards the increase does not accompany the increase in wet weight and the rate of accumulation decreases. From D55 to D87 this occurs in all groups at a similar extent.

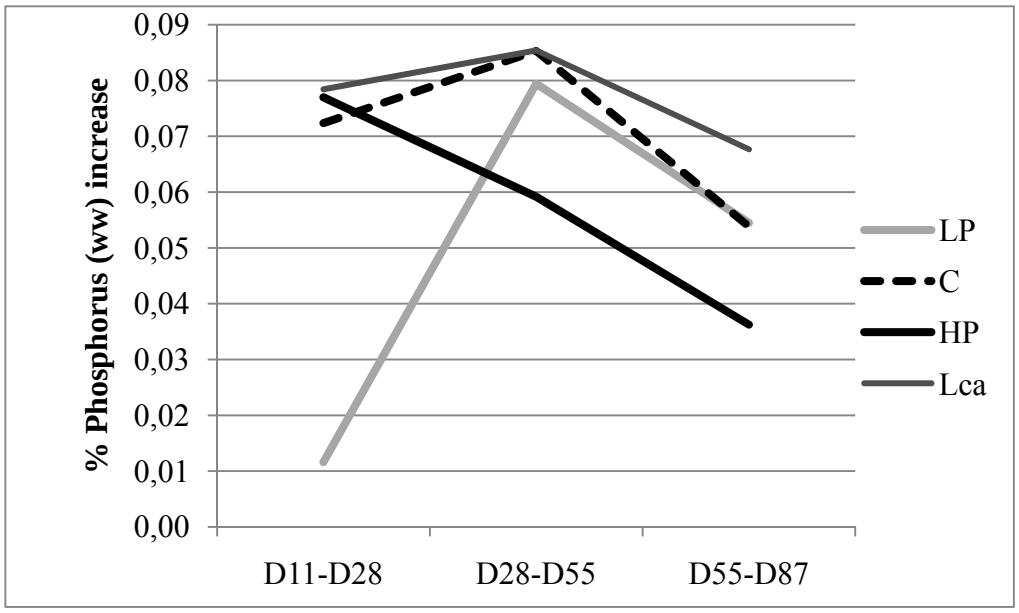

Figure 3.4 - Gain of phosphorus (% per wet weight) between sampling points

Phosphorus utilization

Efficiency of P utilization (% measured P/ingested P) was evaluated but was overestimated especially at the beginning of the feeding trial as it was calculated with distributed food and not absolute values for ingested food. However, apparent efficiency of P utilization was different between the four dietary groups and linked to available dietary P level (Figure 3.5).

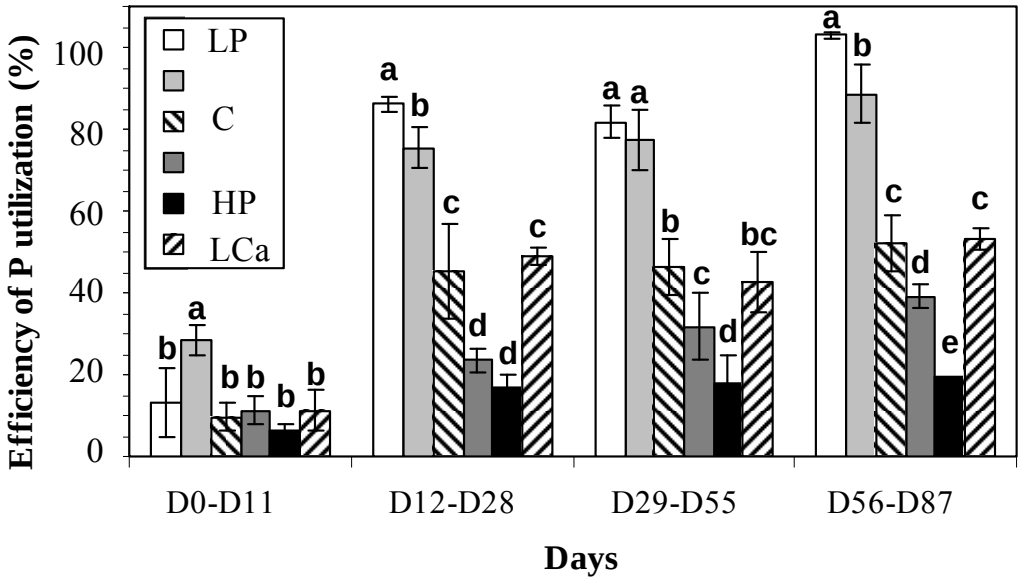

Fig. 3.5 - Efficiency of phosphorus utilization of rainbow trout fry fed the six diets supplemented with different levels minerals. Values are means of 3 rearing tanks except for data of dietary group E: 2 rearing tanks for D29-D55 and 1 rearing tank for D56-D87. Means not

sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by a Newman–Keuls test.

From D0 to day 11 P retention was similar in all treatments, about 10%, from D12 to D28 and increased dramatically reaching almost 90% in LP group around 50% in LCa group and only 20% in HP group. From D29 to D55 the retention levels were very similar to the previous timeframe. During the period D56 to D87 the retention increased again in the group fed the LP diet reaching 100%. This increased retention may indicate that in normal situations the uptake of phosphorus from water is negligible, because the availability of P in fresh water is low (total phosphorus concentration varies between 10 – 50 μg/L, (Wetzel 2001), these individuals may have gained more efficient mechanisms of P absorption through water.

In summary, phosphorus retention decreased with increasing dietary P level and there was no significant effect of dietary Ca supplementation (LP or C) on the efficiency of P utilization.

3.3.3 Skeletal Ontogeny

The development of the axial skeleton and fins was analysed and characterized by the appearance of calcified structures. Classification of skeletal structures as dermal or endochondral bone was based upon their affinity for the histological stains Alcian blue and Alizarin red and revealed that the origin of the skeletal elements studied did not differ from that seen in other teleosts. The ontogeny of trout skeletal development was linked to both with age and standard length although in agreement with previous studies developmental status was more closely linked to standard length ((Faustino and Power 1998, 1999; Campinho et al. 2004). Figure 3.6 is a scheme representing the ossification sequence observed in trout throughout the experiment at 51dpf, 62 dpf and 79 dpf, which corresponds to D0, D11, and D28 days post first feeding respectively. After D28 the ontogeny of the skeleton was almost complete and so qualitative observations were not performed in groups D55 and D87. The fry fed the control diet (C) was taken as the reference for the normal pattern of ontogeny, bearing in mind that the sampling was not continuous but occurred at large time intervals and gave a general overview of skeletal development but did not permit a detailed analysis.

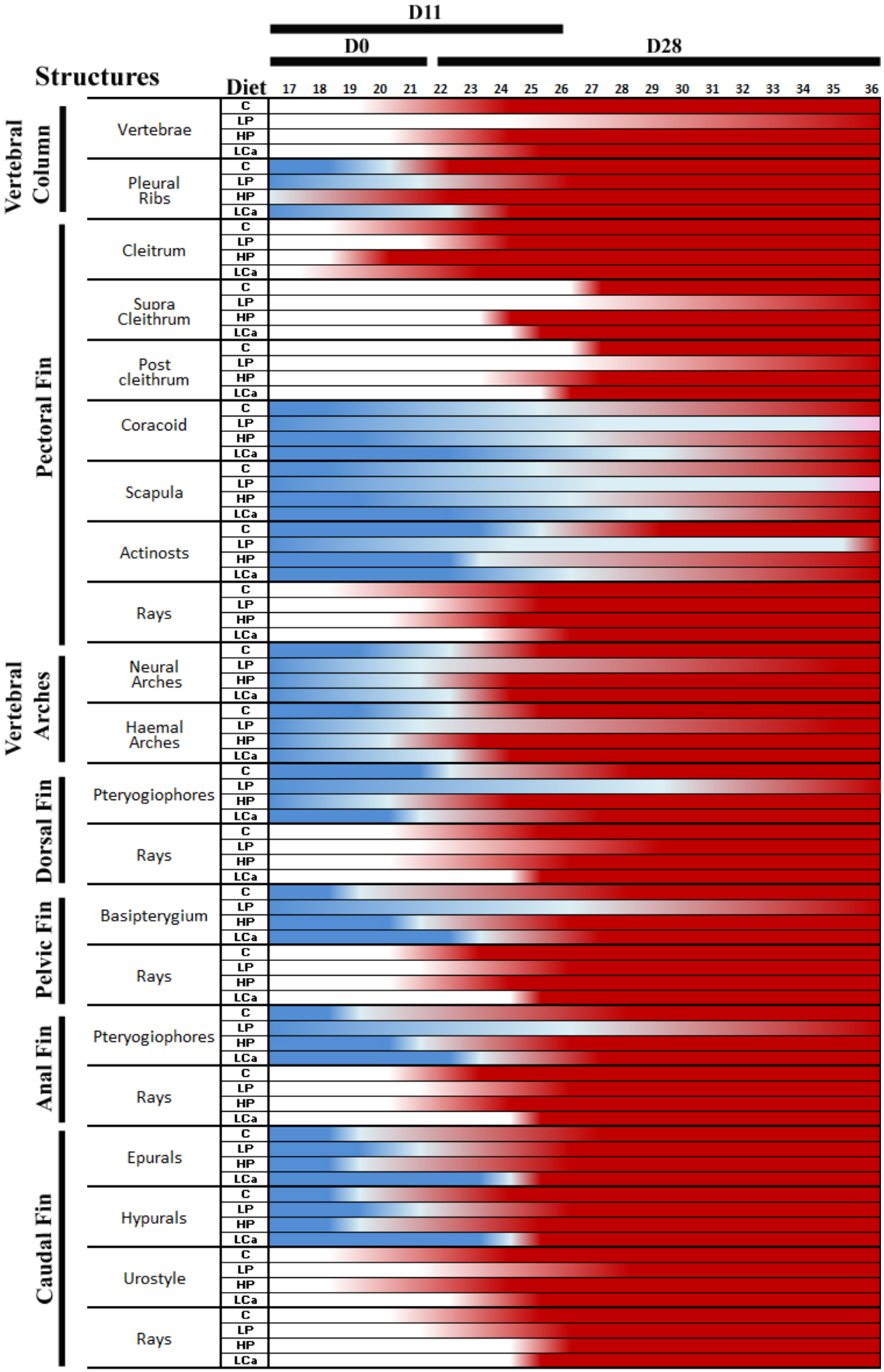

Figure 3.6 - Diagram representing the sequence of ossification in the axial skeleton and fins of *Onchorynchus mykiss* for all the experimental groups LP, C, HP, LCa regarding their standard

length at sampling points D0, D11 and D28. Structures are anatomically subdivided. Full blue indicates the structures are cartilaginous and are present in all individuals with that SL, fading blue indicates the transition period in endochondral structures, fading red indicates the beginning of ossification for some of the individuals with that SL and full red indicates that all individuals with that SL present that structure ossified.

Dermal structures first appear at D11 and the first dermal structure to begin ossification is the cleithrum at around 18.5 mm followed by the urostyle and rays of the pectoral fin at 19 mm. Vertebrae start their ossification at around 20 mm in the vizcranial region and continue in an anterior-posterior direction and all the individuals at 24 mm had varying degrees of ossification in all their vertebrae. At 21 mm the rays from the remaining fins start to ossify and the last dermal structures to ossify in the axial skeleton and fins are the supra-cleithrum and post-cleithrum that is present in all individuals at 26.5 mm.

Some endochondral structures were already present in D0 and were cartilaginous and included the pleural ribs, neural and hemal arches of the vertebral column complex, the coracoid, scapula and actinosts that form the pectoral fin, and the pterigyophores, basipterygium, hypurals and epurals that are part of the other fins. The first of these structures to ossify were the pleural ribs that in group C began their transition at 19 mm and by 22mm they were completely ossified in all individuals. The coracoid and the scapula begin their transition to bone relatively early in development around 20 mm, but this structure has the longest transition period of the structures studied and prolongs until D28, and even in individuals of 33mm they are still not completely ossified. On the other hand the actinosts that also belong to the pectoral fin complex begin ossifying at 23 mm and by 29 mm they are totally ossified. The hemal and neural arches have an anterior-posterior pattern of development, following the vertebra development at 20 mm they start transition to bone and by 24 mm all the individuals have ossified arches. The pterigyophores from the anal fin, the basipterygium from the pelvic fin, the hypurals and epurals start transition to bone at around 18 mm, but the hypurals and epurals ossify quicker and at 26 and 24 mm respectively all samples were ossified, while in the case of the pterigyophores and the basipterygium completion occured only at 27 mm. The pterigyophores of the dorsal fin started transition to bone in individuals of 21mm and by 27 mm were ossified in all the individuals. Modification in the mineral content of the diet significantly affected the ontogeny of the skeleton from that of trout fed compound diet C. Comparison of individuals fed the LP diet to the C group revealed delayed

ossification, which was evident by D11 and was evident for both dermal and endochondral structures and was represented by a prolonged transition period. There was also a small delay in the beginning of transition from cartilage to bone but the most significant delay was observed for the beginning of ossification.

In the endochondral structures transition to bone was observed by the loss of blue staining characteristic of cartilage, some of these structures became almost transparent before they started to acquire the pink and then typical red staining of calcified tissue. In the group fed with low calcium LCa there was a delay in the transition of cartilage to bone and in the calcification process but in the majority of structures transition occured at a similar time to group (C) fed the compound diet. The individuals that were fed HP had an ossification pattern similar to those of the compound diet.

3.3.4 Cumulative counts of skeletal structures

In order to transform the qualitative analysis of skeletal ontogeny into a quantitative analysis, cumulative counts of structures were made subdividing them by bone category and degree of ossification. The results are depicted in Figure 3.7 and 3.8.

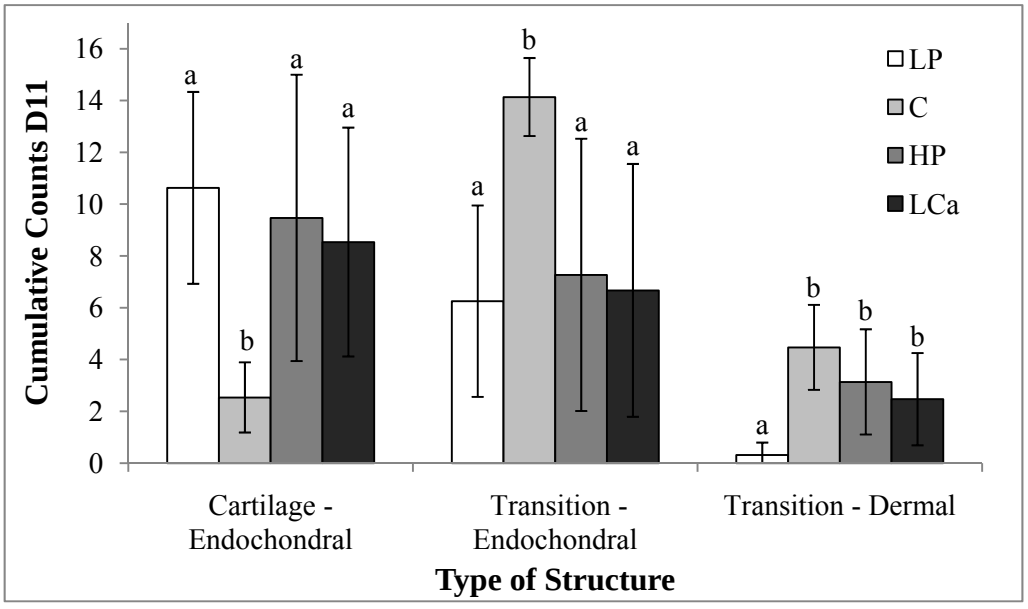

Figure 3.7 - Level of ossification in the different structures of rainbow trout fry fed the selected diets LP, C, HP, LCa with different mineral content and sampled on day 11 of the experiment. Means not sharing a common superscript letter are significantly different (P<0.05) according to one-way ANOVA followed by a Newman-Keuls test (average±SD).

By D11 group C had on average a greater number of both dermal and endochondral structures in transition, than in individuals fed the modified diets, this difference is statistically significant for all groups in endochondral structures and for LP group in dermal structures (Figure 3.7).

Interestingly after 1 month of exogenous feeding (D28) no significant differences in ontogeny of endochondral or dermal structures was found between trout fed the control diet and diets with an increased P content despite differences at D11, suggesting that an excess P enables a quicker transition to bone for endochondral structures in these individuals (Figure 3.8). In contrast, a persistent modification of dermal and endochondral ontogeny was still evident one month (D28) after feeding trout diets deficient in P and Ca.

The delay in ossification of endochondral structures was reflected by the significantly lower cumulative counts of ossified structures in trout fed diets poor in P and Ca compared to trout fed the control diet, although this difference was only significant for the LP group as many of the structures of group LP had a prolonged transition to bone and differed significantly from other groups.

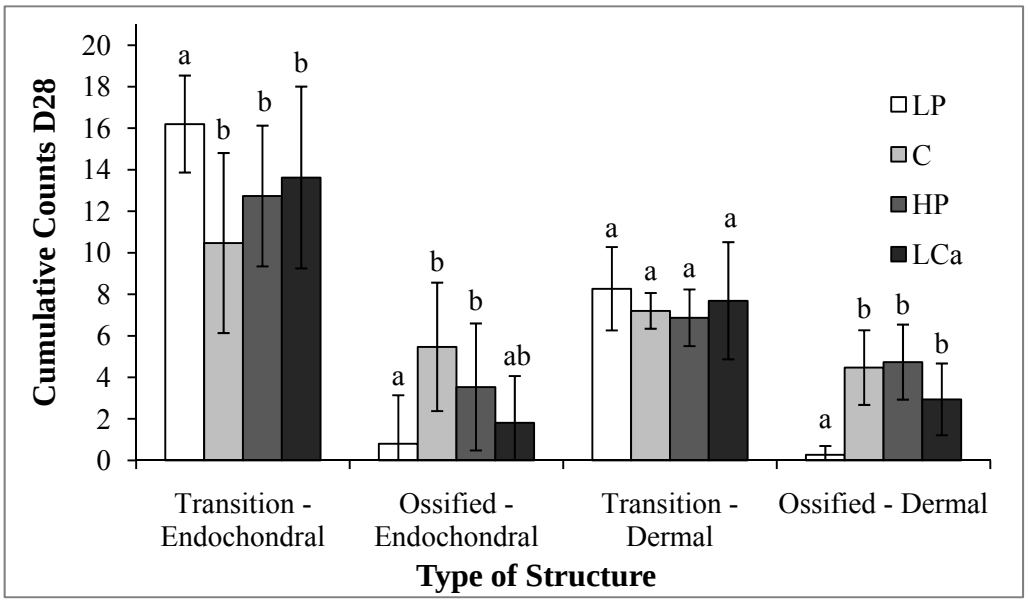

Figure 3.8 - Level of ossification in the different structures of rainbow trout fry fed the selected diets LP, C, HP, LCa with different mineral content and sampled on day 28 of the experiment. Means not sharing a common superscript letter are significantly different (P<0.05) according to one-way ANOVA followed by a Newman-Keuls test (average±SD).

Although no significant differences in cumulative counts of transitional dermal structures were recorded between trout fed modified diets and the compound diet, there was a significant difference in the number of cumulative counts of ossified dermal structures in the LP group compared to all the others.

3.3.4 Morphometric Analysis

In order to establish a connection between skeletal development and the morphometric properties at an age where the skeletal ontogeny is established, some measurements were made in individuals from D55 and D87.

Number of vertebra

The total number of vertebra in individuals with completed skeletal ontogeny from ages D55 and D87 varied between 59 and 64 vertebrae depending on the individual. The average number of vertebra was not significantly affected by diet and the majority of the individuals had 61, 62 or 63 vertebrae (Figure 3.9). Moreover, the frequency distribution of vertebra number was modified by diet with the distribution shifted to the right in the case of fish fed HP diet, with a tendency to have an increased number of vertebra and individuals from diet LP had a less variable number of vertebra.

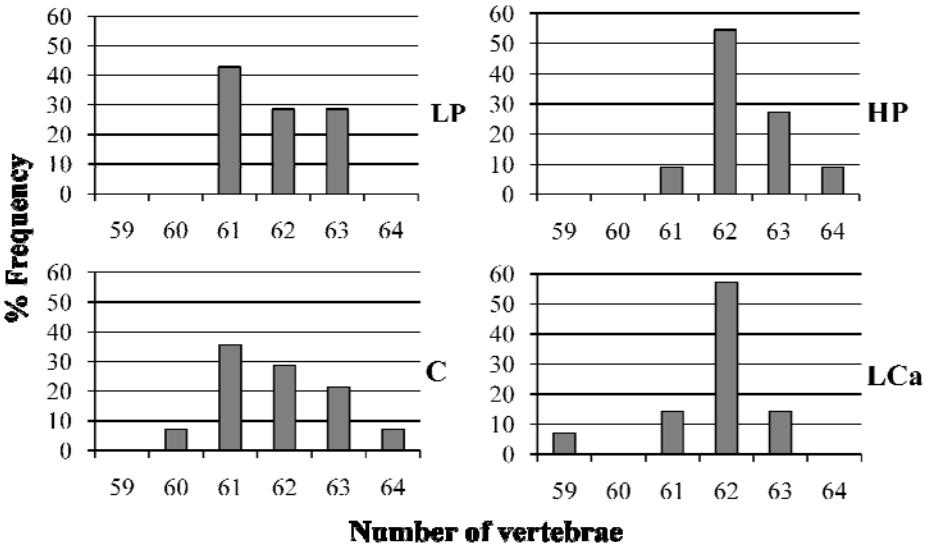

Figure 3.9 – Frequency plots of the number of vertebra present in the fully formed vertebral column of trout, at sampling points D55 and D87, fed with experimental diets.

Morphometry of vertebrae

As observed previously (Kacem et al., 2003) the morphology of vertebrae along the column was variable and the column was divided into 4 main regions based upon their distinct morphologies. The vertebrae are wider in the trucocaudal region and narrower in the caudal region. Analysis of the length, height and area of vertebra and intrerneural arch space in the mid-region of the 4 main areas of the column identified revealed that the effect of feeding a modified diets for 87 days on the vertebral structure in rainbow trout (Table 3.4).

The LP diet had no significant impact on vertebra measurements compared to those of trout fed the C diet (Figure 3.10).

Table 3.4 - Length, height, intervertebral length and area of 3 individual vertebra located in the mid-point of each of the 4 regions of the vertebral column in trout (D87) fed diets with a modified mineral content (n = 9 for diet LP, C and LCa and n = 6 for diet HP).

Column Region	Diet	Vertebral Height (mm)	Vertebral Length (mm)	Intervertebral Length (mm)	Vertebral Area (mm2)
Vizcranial	LP	0.88±0.10^{a}	0.48±0.07^{a}	0.58±0.1^{a}	1.32±0.29^{ab}
	C	0.82±0.08^{ab}	0.51±0.06^{ab}	0.65±0.12^{ab}	1.33±0.16^{a}
	HP	0.87±0.13^{a}	0.57±0.08^{b}	0.69±0.1^{b}	1.54±0.27^{a}
	LCa	0.76±0.14^{b}	0.48±0.08^{a}	0.65±0.07^{ab}	1.13±0.27^{b}
Truncocranial	LP	0.91±0.10^{a}	0.57±0.08^{ab}	0.73±0.10^{ab}	1.65±0.36^{a}
	C	0.85±0.08^{ab}	0.59±0.09^{a}	0.73±0.10^{ab}	1.57±0.29^{a}
	HP	0.90±0.13^{a}	0.64±0.11^{a}	0.80±0.13^{a}	1.80±0.32^{a}
	LCa	0.75±0.15^{b}	0.51±0.10^{b}	0.69±0.09^{b}	1.22±0.36^{b}
Truncocaudal	LP	0.95±0.11^{a}	0.64±0.09^{ab}	0.84±0.11^{ab}	1.9±0.38^{a}
	C	0.94±0.11^{a}	0.68±0.09^{a}	0.85±0.11^{ab}	2.01±0.39^{a}
	HP	1.00±0.18^{a}	0.68±0.14^{a}	0.89±0.14^{a}	2.13±0.44^{a}
	LCa	0.84±0.15^{b}	0.59±0.14^{b}	0.78±0.11^{b}	1.55±0.46^{b}
Caudal	LP	0.81±0.11^{a}	0.42±0.07^{a}	0.65±0.10^{a}	1.08±0.26^{a}
	C	0.76±0.09^{ab}	0.46±0.09^{ab}	0.65±0.12^{a}	1.11±0.26^{a}
	HP	0.81±0.12^{a}	0.51±0.13^{b}	0.73±0.13^{a}	1.3±0.36^{a}
	LCa	0.72±0.09^{b}	0.46±0.08^{ab}	0.66±0.10^{a}	1.05±0.24^{a}

Within columns, means not sharing a common superscript letter are significantly different (P<0.05) according to one-way ANOVA followed by a Newman-Keuls test (average±SD).

In contrast, HP diet significantly increased the area of vertebra in all the regions, although it was not possible to detect a significant change in the height and width of vertebrae. The results appear to indicate that phosphorus enriched diets tend to cause an overall increase in vertebral size compared to the control diet (Figure 3.10).

In trout fed LCa diet persistent modifications were associated with vertebrae in all body regions. There was a reduction in the height and area of vertebrae and these differences were statistically significant. Intervertebral length and vertebral length were also reduced. These morphometric results in LCa group can account for the tendency of decreased growth depicted in Table 3. 1. It would appear that a reduction in dietary calcium in developing trout leads to a reduction in vertebrae size and probably in the long term to decreased growth rates.

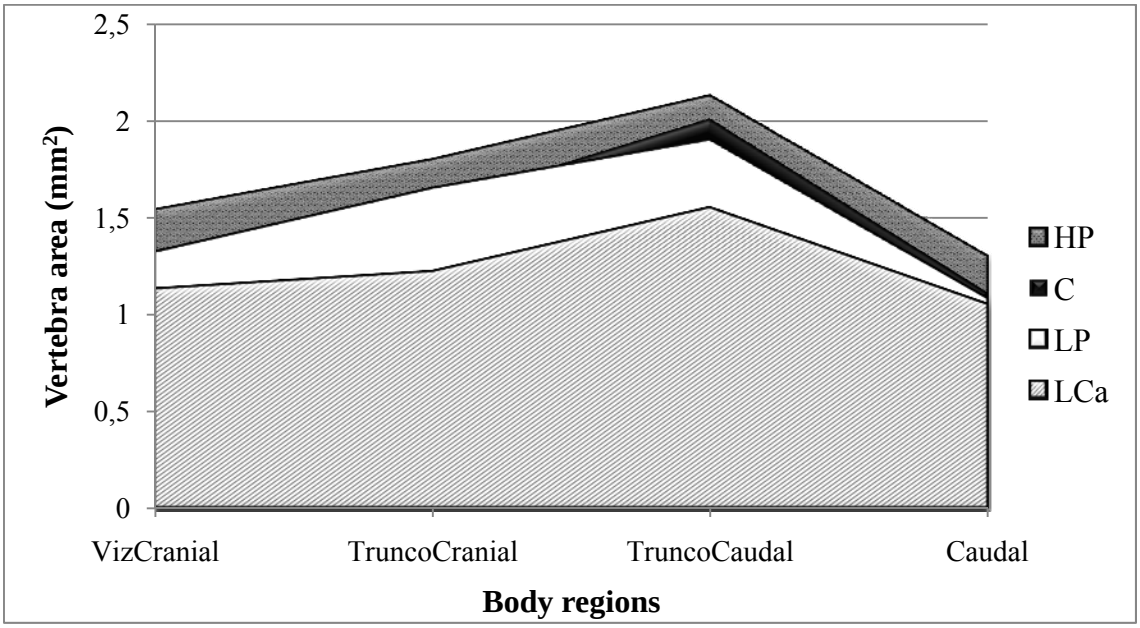

Figure 3.10 – Mean area (mm^2) of 3 individual vertebra located in the mid-point of each of the 4 regions of the vertebral column in trout (D87) fed diets with a modified mineral content

When the overall pattern of vertebra area is plotted for the different vertebral column regions (figure 3.10) it is evident that the HP group had the largest vertebra in all regions, that the LP and C groups were very similar and that LCa had the smallest vertebra in all the regions. It seems that the lack of availability of calcium in the diet compromised the size of the vertebrae and that this does not seem to happen with the low phosphorus diet.

Ossification level of the vertebral column

In order to determine whether if in addition to anatomical modifications the degree of ossification was modified, the mean grey value was measured. In Figure 3.11 it is patent that diet LP although it did not cause significant differences in the size of vertebra they had significant lower mean grey value (proxy for ossification) in TCr, TCa and Ca regions. In contrast, LCa individuals which had smaller vertebrae in most of the vertebral column regions (Table 3.4) did not have a significant change in ossification level compared to the C group. The HP group did not differ from the C group although the mean grey value levels are higher in VCr and TCr regions.

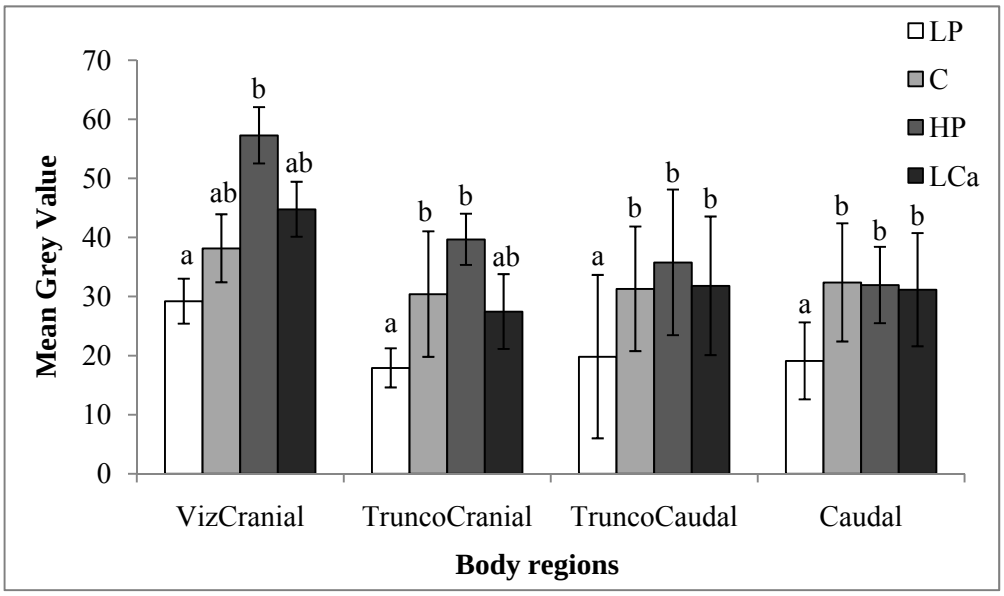

Fig. 3.11 - Whole body autoradiographs (n=9 per experimental group) were used to determine the mean grey value (a proxy for density) of five vertebra in each of the main regions of the vertebral column. Means not sharing a common superscript letter are significantly different (P<0.05) according to one-way ANOVA followed by a Newman-Keuls test. (average±SD).

Although the mean grey value was used to quantify the degree of calcification, cursory observation of the X-ray pictures (Figure 3.12) made differences in ossification immediately evident.

LP vertebras seem almost hollow and the extremities less bright, while LCa are clearly shorter but the core of the vertebra is white. HP group in this case have the biggest and whitest vertebra even when compared with the C group.

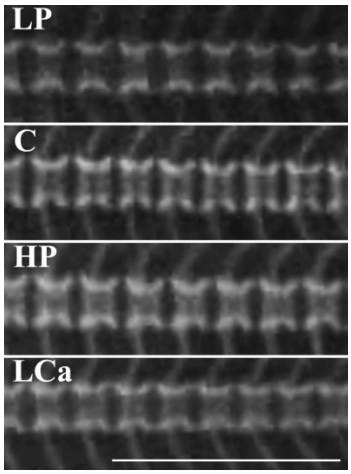

Figure 3.12 – X-ray of the truncocaudal region of trout depicting the differences in morphology and degree of calcification of individuals fed with diets containing different mineral levels (LP, C, HP, LCa)

3.3.5 Malformations at D11, D28 and D55

At age D11, D28 and D55 no external malformations were observed except for some kyphotic behavior at D55, and internal malformations were identified by pre-staining the fish with cartilage/bone whole mount stain (see part 3.2). The general incidence of anomalies in these ages was calculated (Figure 3.13) as well as the percentage incidence of each type of malformation in each experimental group (Figures 3.14 and 3.15).

Incidence of malformations

The incidence of malformation at D11 was relatively low for all groups 20 – 30%, but at D28 when there were more structures evident the incidence increased tremendously in LP and LCa groups to 110% and 80% respectively and only slightly in HP group whereas in C group the incidence decreased slightly. At D55 it seemed that some of the anomalies detected in LP and LCa disappeared and the incidence decreased to 80 and 60% respectively whilst the C and HP group increased to 100% and 80% respectively.

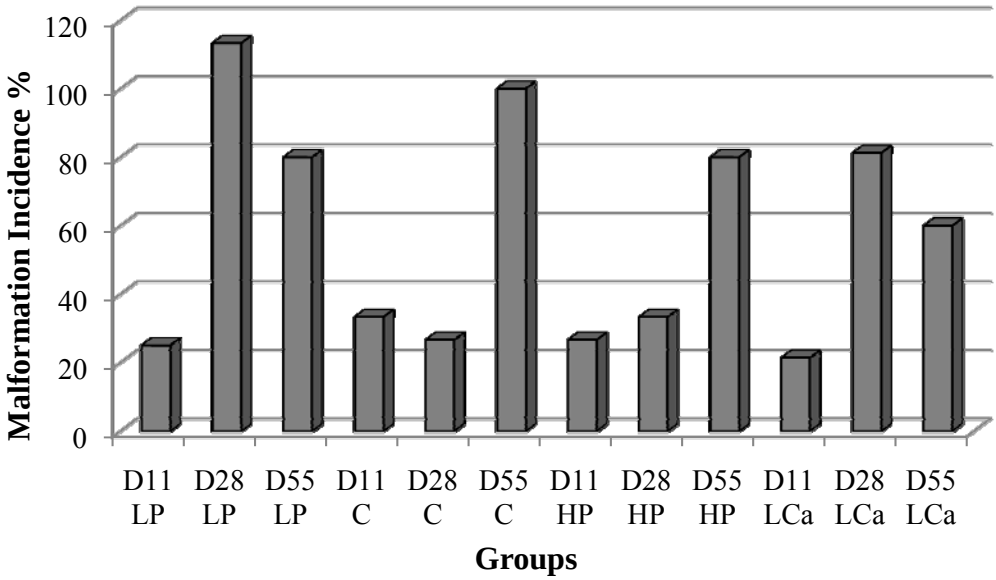

Figure 3.13 - Malformation incidence (total number of malformations in the group/number of individuals in %) in the experimental groups.

Type of malformations D11, D28

The main categories of anomalies present at D11 were concentrated in the caudal, anal and dorsal fins and vertebral arches, whilst at D28 they were mainly associated with caudal fin, vertebral arches, pleural ribs and vertebra. At this developmental stage differences in the type of malformations present in each group were already patent (Figure 3.14), although sometimes these differences were more related to the absence of structure which was still ossifying.

Anomalies of the caudal fin complex consisted in malformations and variations in the number of epurals and hypurals and the greatest incidence occurred in LP group at D28. Pleural ribs were often wavy or twisted and were only visible after complete ossification, which happened at D28, this anomaly had a greater incidence in LCa D28 and was inexistent in C D28. The vertebrae were sometimes fused, this happened mainly in group LP D28 but also in LCa D28. Dorsal and anal fins present twisted or fused pterigyophores only detected at D11 and were present in LP and C. Vertebral arches were twisted or fused and were present in all groups and ages, but the incidence is higher in LP and LCa at D28.

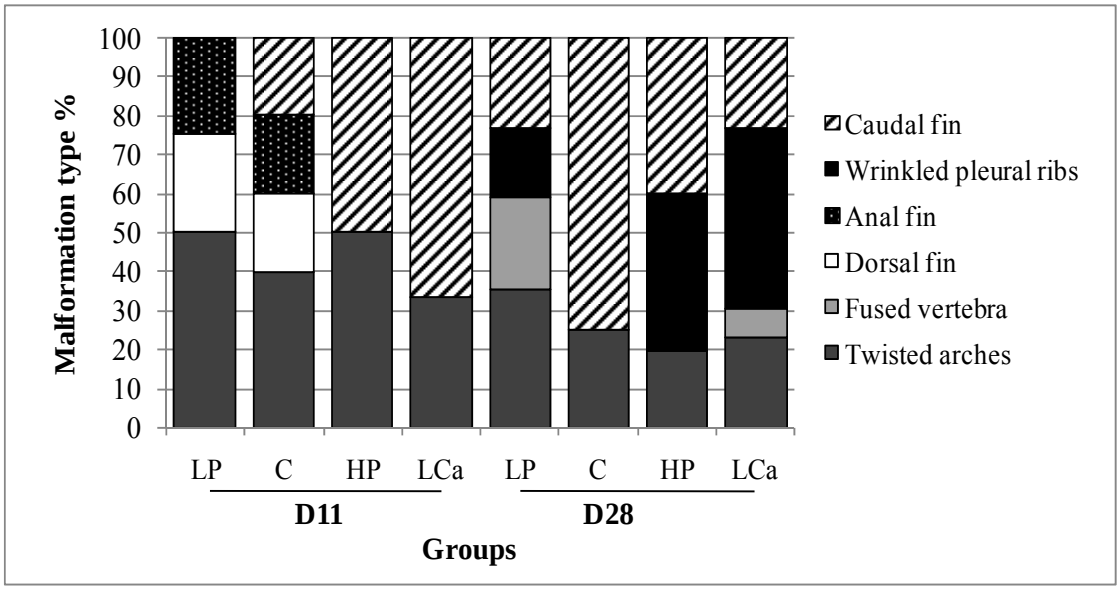

Figure 3.14 - Incidence of malformations within experimental groups at D11 and D28, in which the frequency of each abnormality is considered as a proportion of the total number of abnormalities (100%) detected per experimental group.

Type of malformations D55

The external analysis of malformations of the animals at D55 showed the presence of kyphotic behavior in the vertebral column in some individuals, no other type of external abnormality was detected, in the cranium, jaws or operculum the site of common malformations in aquaculture species. After staining for cartilage/bone internal malformations were identified and there was a high incidence of abnormalities in all the groups (Figure 3.15).

At this age the malformations were subdivided in 5 categories: kyphosis, in which the fish body presents a slightly arched shape and this, was more frequent in LCa group. Wrinkled pleural ribs were only noticed in group C. Vertebral compression was present in group LP and had the highest incidence in HP, and was characterized by a narrower intrerneural arch originating what appeared to be the initiation of fusion of some vertebra. LCa was the only group that has fused vertebra while twisted vertebral arches were common in LP and C and were also present in HP.

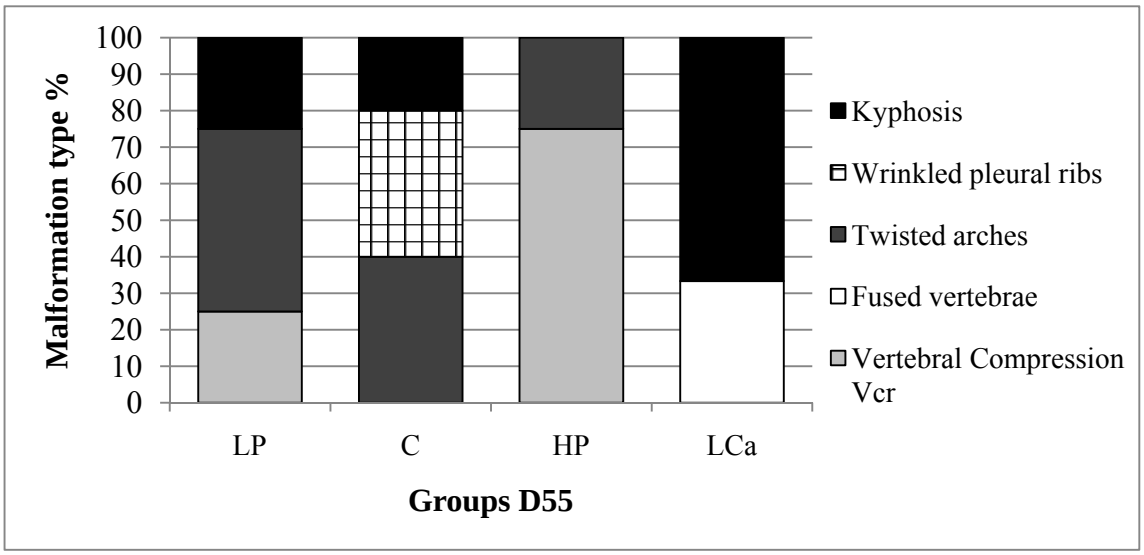

Figure 3.15 - Incidence of anomalies within experimental groups at D55, in which the frequency of each abnormality is considered as a proportion of the total number of abnormalities (100%) detected per experimental group.

3.3.7 Malformations at D87

The external analysis of malformation of the animals at D87, showed the presence of kyphotic behavior in the vertebral column of some individuals, no other type of external abnormality was detected. Analysis of the skeleton by X-ray allowed more subtle malformations to be detected which do not affect the external fish morphology. During this experiment there was a very high incidence of individuals that showed some kind of anomaly (Figure 3.16)

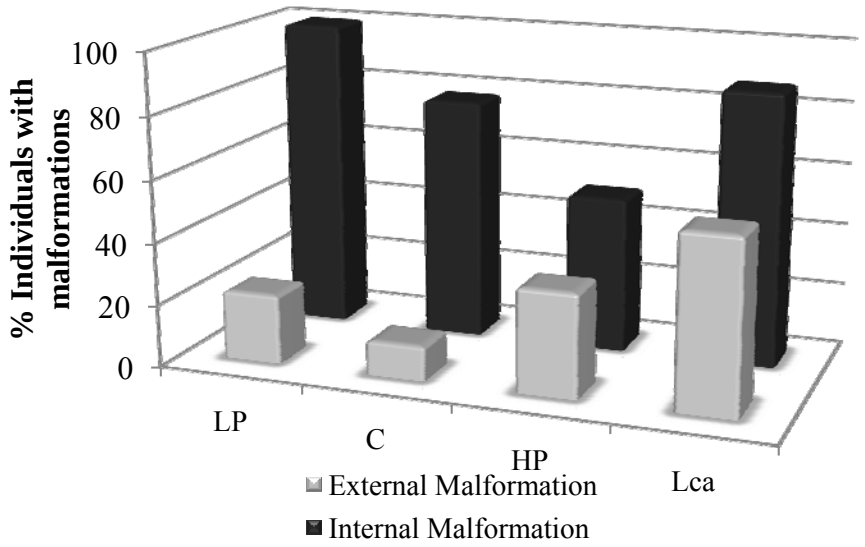

Figure 3.16 - Comparison of the percentage of rainbow trout fed modified LP, HP and LCa or compound (C) diets in which abnormalities were visible as a modification in the external morphology (light bars) and those detected after detailed examination of radiographies (dark bars). Note, a high incidence of internal abnormalities were detected which did not lead to a perceptible change in external morphology.

Type of malformations D87

Figure 3.17 describes the malformations percentage observed at sampling point D87, there is not a great variability in the type of malformations present and these can be grouped into 6 categories: irregularity in vertebrae disposition throughout the vertebral

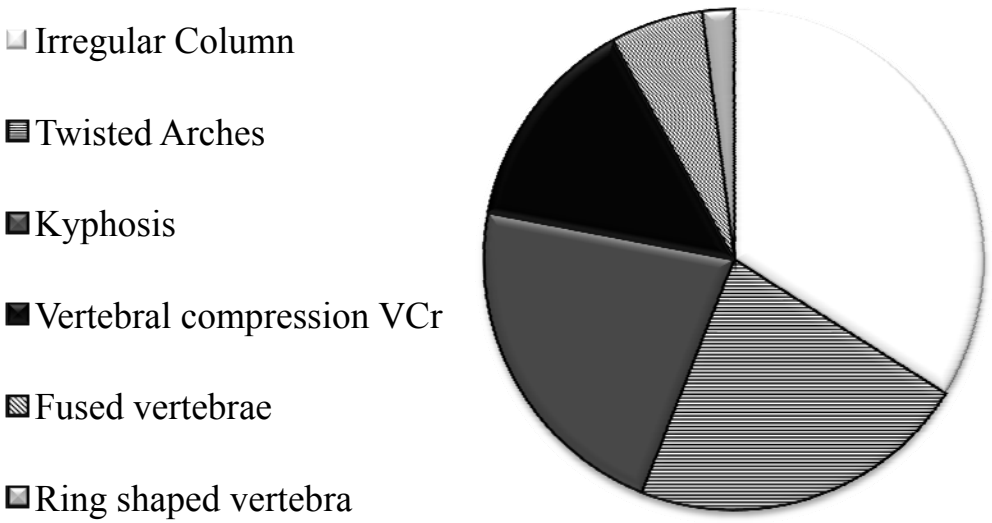

Figure 3.17 – Incidence of the malformations observed in trout in all experimental groups at D87.

column was the most frequent malformation followed by twisted neural and hemal arches and kyphotic behavior of the column in the vizcranial region; the fourth most common malformation was compression of vertebrae in the vizcranial region followed by fusion of vertebras in the caudal region and the presence of ring shaped vertebrae in the vizcranial region.

Illustrations of each category of malformation are represented in figure 3.18. The deviation from linearity observed throughout the column was named irregular column. Its origin seemed to be associated with vertebral slipping which was characterized by 1 or more vertebrae appearing to be slightly above or below the axis of the column

(arrowheads in A). Wrinkled or twisted hemal and neural arches (arrows in B) were present throughout the vertebral column and were not necessarily related with other types of abnormalities also occurring in the vertebrae

Fused vertebrae were predominantly present in the caudal region, the fusion of only 2 vertebra was the most common situation (arrowheads in C). Ring shaped vertebrae were present in the vizcranial region; these possessed a typical shape and had a reduced intrerneural arch (D). Vertebral compression/deformation (E) was present in the vizcranial region and was characterized by the virtual absence of intervertebral space and by the distortion of vertebrae that did not have a characteristic X shape and originated fusion (complete or partial) of some vertebra.

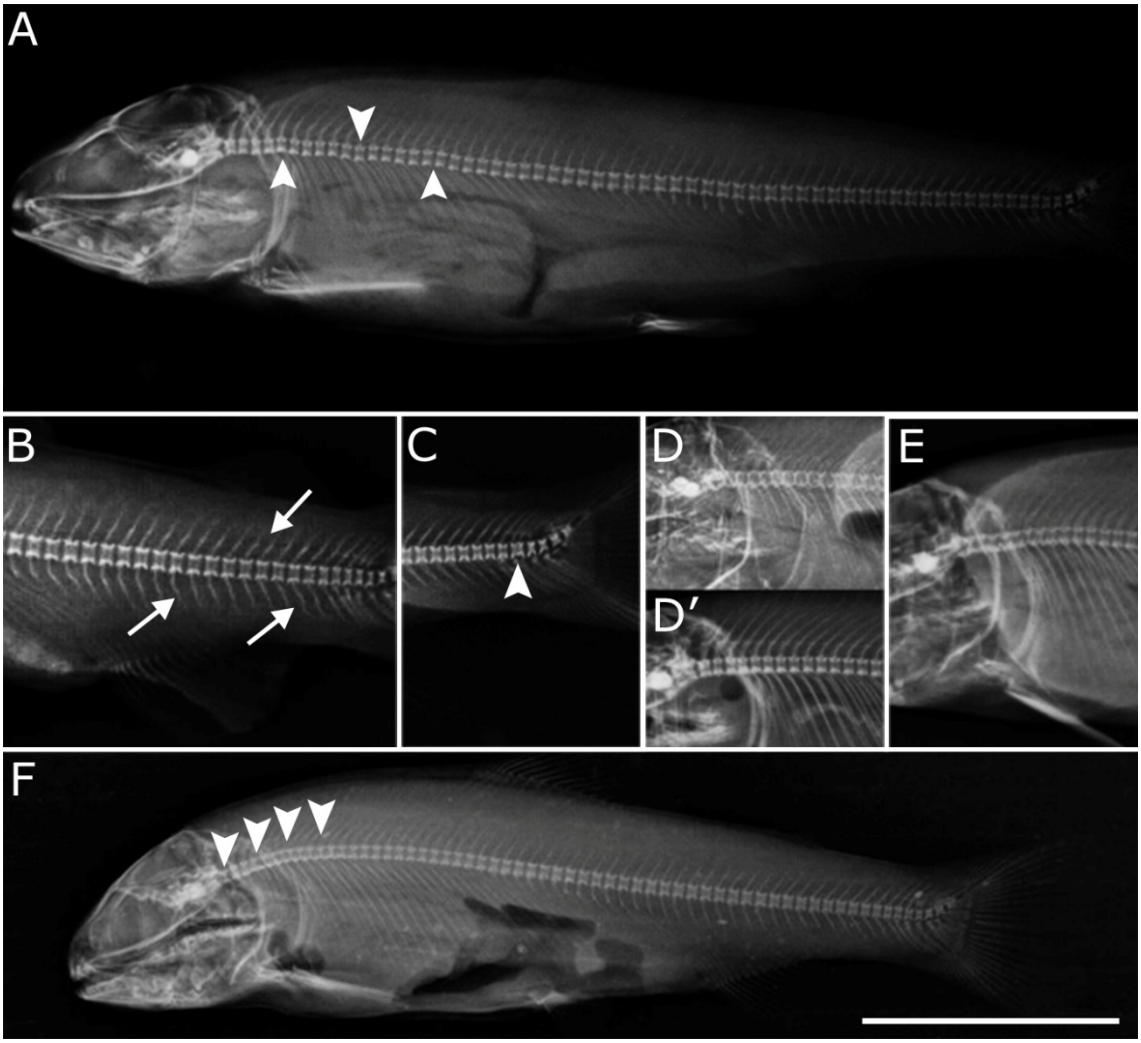

Figure 3.18 - Rays representative of the malformations present in individuals from age D87. A - Irregularity in vertebrae disposition in throughout the vertebral column. B - twisted neural and heamal arches. C - Fusion of vertebra in the caudal region (note, that 2 haemal and neural arches are associated with a single vertebra). D - Ring shaped vertebrae in the vizcranial region. D'- Appearance of a normal vertebral column in the vizcranial region. E - Compression of vertebrae

in the vizcranial region. F - Kyphotic behavior of the column in the vizcranial region. Scale bar 2cm.

The kyphotic behavior of the column (arrowheads in F) was, in many cases but not exclusively, related with vertebral compression/deformation in the vizcranial region, and when this happened the number of affected vertebra reflected the gravity of the kyphosis observed externally.

The incidence of anomalies varied with the type of diet that was administered as is evident from Figure 3.19. With diet LP, C and LCa the most frequent anomaly was an irregular column, this type of abnormality is not usually described in the literature but was persistent in all the groups studied. These groups also had a relatively high incidence of twisted arches. Ring shaped vertebrae were only observed in the LCa group. Fused vertebrae were only detected in groups C and HP. This last group, HP, had the lowest overall incidence of abnormalities which were mainly compression of vertebrae in the vizcranial region and kyphotic behavior.

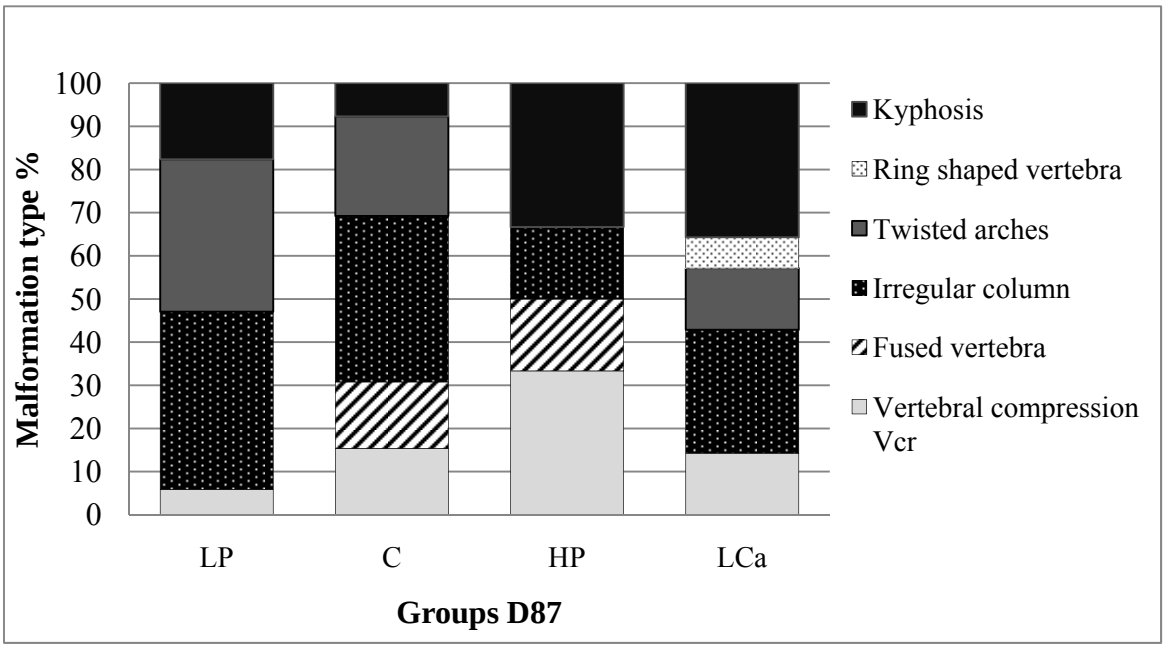

Figure 3.19 - Incidence of anomalies within experimental groups at D87, in which the frequency of each abnormality is considered as a proportion of the total number of abnormalities (100%) detected per experimental group.

At D87 it was not possible to confirm the malformations in the fins observed at earlier stages (D11, D28 and D55) as these bones were not visible with X-ray.

3.4 Discussion

In this experiment the effect of lowering the content of phosphorous and calcium and increasing the amount of phosphorus in the diet of first feeding trout for 87 days was analyzed.

None of the experimental diets caused a significant effect on growth, neither in weight nor in length. Previous studies have demonstrated the effect of a low level of P in diets of several aquatic organisms (Brown et al. 1993; Dougall et al. 1996; Skonberg et al. 1997; Baeverfjord et al. 1998; Velasco et al. 1998; Chavez-Sanchez and Ross 2000; Sugiura et al. 2000; Sugiura and Ferraris 2004a; Sugiura et al. 2004b; Ye et al. 2006; Uyan et al. 2007) the results vary according to the species, or the type of experiment performed but reduced growth rate was characteristic of all studies. In contrast, administration of modified diets to fingerling gave different outcomes, in trout (initial weight 1.8g) P deficiency did not limit growth after 8 weeks (Skonberg et al. 1997), in salmon (initial weight 4.7g) the growth was affected only after 9 weeks (Baeverfjord et al. 1998) of feeding a P restricted diet. This could mean that, the low calcium group (LCa) was absorbing sufficient calcium from the environment, and that the amount of P supplied to the low phosphorus (LP) group was enough to cover the needs for growth. Previous results state that salmonids may be in a phosphorus-deficient state well before a reduction in weight gain is significant (Shearer and Hardy, 1987). Baeverfjord and Shearer (1998) refer to this situation and indicate that salmon have a homeostatic mechanism to manage shortages of P which involves its mobilization from mineralized skeletal structures. P restricted fish also increased the efficiency of P uptake from food and between 80 and 90% was utilized showing a highly efficient mechanisms of recovery. Increased efficiency of P absorption has been demonstrated in adult trout fed a P deficient diet and was associated with an increase in the expression of genes involved in P absorption and metabolism ((Sugiura et al. 2000; Kirchner et al. 2007; Sugiura et al. 2007)Sugiura and Ferraris 2004a) and this will be explored in a subsequent chapter.

Analysis of skeletal ontogeny showed that P restriction limited the rate of skeletal development of both endochondral and dermal structures. The modification in the skeleton when P is restricted was also reflected in the mineral and ash content of the

body which decreased throughout the duration of the experiment and by the end of it the bone density was significantly decreased in these individuals. The skeleton formed in trout fry fed the P deficient diet and the vertebral centra was not significantly smaller than in trout fry fed the control diet, but the magnitude of calcification was lower and the bone was less dense. The reduced density of bone in trout fry fed P deficient diet but with the same rate of growth as trout fed control diets was associated with an increased incidence of deformities, and this probably resulted from the load on weakened bones. The anomalies detected have already described in hypophosphatemic fish and included twisted vertebral arches but also vertebral fusion and vertebrae slippage ((Takagi and Yamada 1991; Skonberg et al. 1997; Lellis et al. 2004; Cheng et al. 2006; Ye et al. 2006)Lall and Lewis-McCrea 2007). Factors which could contribute to these effects are the deceleration of bone mineralization due to an increase in the amount of osteoid and number of osteoclasts and a decrease in the number of osteoblasts described for hypophosphatemic fish (Takagi and Yamada 1991; Roy 2002). Histological and histochemical examination of P deficient haddock also showed an initial increase in bone resorption, which was subsequently followed by a decrease in bone mineralization and reduced bone formation (Roy and Lall 2003). These observations based on the increase in the number of osteoclasts suggest that osteoclasts may be involved in P homeostasis, by rescuing phosphorus from the bone for primary metabolic necessities.

Analysis of variance indicated that LCa diet had no significant effect on growth or body composition, which is in agreement with the generally accepted view that most fish can absorb Ca from the aquatic environment to meet their requirements (Ye et al. 2006). In fact the concentration of dietary calcium rarely seems critical for salmonids, and a dietary requirement has not been demonstrated (NRC 1993b). Moreover the experimental diets were casein-dextrin based, and contributed 0.1% calcium to the LCa diet. This amount appears to cover most of the calcium needs for the LCa group to obtain normal growth and ossification, at least at an early stage, and is probably supplemented with Ca absorbed from the water. However, by the end of the experiment the growth rate of these individuals started to decrease. Suggesting that if the experiment had been prolonged a probable growth impairment would be noticed and indicating that despite the availability of Ca in water (83 mg/L in the experiment) there is a dietary requirement for Ca in trout fry.

Calcium restriction also had an effect on bone development by decreasing slightly the rate of calcification of the skeleton at D11 and D28. The effect is not as striking as for the P restricted animals and is transient as proved by the analysis of bone density at the end of the experiment which was similar to the control group. Nevertheless although bone density is not affected the size of the vertebrae centra is smaller showing that Ca restriction affects the amount of bone formed, in this case the vertebrae were smaller but presented the same bone density as control. Typical deformities of dietary Ca restriction include curved spines and vertebral compression ((Copp et al. 1962; Chavez-Sanchez and Ross 2000)Baeverfjord et al. 1998; Lall and Lewis-McCrea 2007) and were observed in some specimens in the present study, but in addition a high incidence of kyphosis visible externally was observed in 65% of the individuals. In fact LCa group presented the highest incidence of externally detected malformations, which is very detrimental from a commercial viewpoint. Prolonging the experiment would therefore be important to establish the long term effect of calcium restriction on growth for the LCa group. The current data raised the question that fish with calcium deficiency may start to display growth reduction only 3 months after first feeding.

Excess of dietary P had one notable effect, 90% mortality. This effect in younger fish as been reported in another experiment when trout of different initial weights receiving 1.20% P diet, and 200-g and 300-g fish experienced high mortality during the first 3–20 days of feeding. This mortality did not occur among fish of 400 g fed a high P diet (Lellis et al. 2004), and has led to the hypothesis that the timing at which the high P diet is given is crucial in determining the outcome. It would be interesting to clarify which mechanisms are present in the individuals with 400g w/w that aren´t already present at first feeding, 200 and 300g trout. The observed increase in mortality could relate more to the form in which P was supplied rather than to P levels per se. In the present experiment P was supplied in the form of sodium and potassium phosphate (1:), trout fed high P level supplied as calcium and potassium phosphates (1:1) displayed only growth depression (Satoh et al. 1993) and not a decrease in survival as seen in experiments were sodium phosphate was used in combination with calcium phosphate (Lellis et al. 2004). We used here a mixture of sodium and potassium phosphates and tested with first-feeding fry which are more susceptible and generally display higher mortality than juveniles. The compromised survival observed could be due to reduced absorption of other essential trace elements at a high dietary P intake; these are thought

to form a complex with P becoming unavailable for metabolism. Both high and low dietary P intakes appear to interact with the utilization of other minerals and nutrients (Lall 2002; Lall and Lewis-McCrea 2007) and could therefore compromise metabolism. The fact that only 10% of the HP individuals survived may explain the increased growth of these animals that exists although it is not statistically different due to the reduced numbers. Tank density was very diminished and this fact is known to influence growth in a positive way (Mäkinen and Ruohonen 1990; Sirakov and Ivancheva 2008). The increased levels of body phosphorus measured at D11 in these animals may be an indication of the disturbance in homeostasis and it was from this day on that the rate of mortality increased dramatically. This happened in spite of the decreased efficiency of P utilization.

Skeletal development of this group was also decreased at an early stage, in a similar way to the calcium restricted group but seemed to recover by D28 to a pattern analogous to the control. To this fact probably contributed the high mortality observed which selected only the fittest larvae to survive and these were probably the ones with the fastest development that adapted easily to the conditions. Several lines of research indicate that Pi works as a stimulus capable of increasing or decreasing several pivotal genes (Sugiura and Ferraris 2004a; Jin et al. 2006; Jin et al. 2007) which could contribute to the successful outcome for the individual. By the end of the experiment this group presented a bone density superior to the control group, a slightly higher level of body calcium and bigger vertebrae, this together with the fact that this group showed the smallest incidence of abnormalities reinforces the idea that a phenomenon of "survival of the fittest" occurred.

Furthermore in mammals, unlike calcium, phosphorus is readily and efficiently absorbed at all levels of dietary process consequently, plasma phosphorus concentrations are more commonly disrupted by oral ingestion in normal individuals(Calvo 1993; Calvo et al. 1996). The knowledge of P excess metabolism would be of great value in the future taking into account the dietary drift towards high protein observed in humans in the last decades.

3.5 Conclusion

In conclusion phosphorus deficiency in initial stages of development of rainbow trout do not compromise growth but delay calcification of the skeleton, decrease bone density, and increase the incidence of malformation. Restriction of dietary calcium in water with a calcium concentration of 83 mg/L transiently delayed the ossification process but did not compromise the degree calcification of fry, although long term restriction may lead to reduced growth in juvenile trout. Excess P instead compromises survival of the larvae, no other study had described this effect so early in development, which is dramatic, and should be taken into account when diets are being formulated. The present experiment reveals how skeletal development can be manipulated by diet and the results highlight a number of questions which will be of interest to study in the future.

PART 4

EFFECTS OF DIETARY PHOSPHORUS AND CALCIUM LEVEL ON ULTIMOBRANCHIAL GLAND AND CORPUSCLES OF STANNIUS IN TROUT (*Oncorhynchus mykiss)* FRY

4.1 Introduction

Mineral metabolism is tetrapods is balanced by several factors, and is mainly regulated in 3 organs: gastrointestinal tract, kidney and bone and is under control of hormones that maintain mineral homeostasis, these include calcitonin, parathyroid hormone and vitamin D.

Control of calcium homeostasis in fish is different from that in terrestrial vertebrates because the aquatic environment, particularly sea water, provides a constant supply of calcium so that mechanisms of internal calcium mobilization and deposition, in which calcitonin is an important factor for tetrapods, are less essential and less well developed (Clark et al. 2002). Bony fish are capable of regulating plasma calcium levels with great precision irrespective of the calcium content in the aquatic environment (Sterba et al. 1993). Several hormones have been suggested to be involved in mineral metabolism including prolactin, cortisol, PTH, PTHrP, stanniocalcin and calcitonin (Bonga and Lammers 1982; Wagner and Dimattia 2006; Guerreiro et al. 2007). A wide variety of factors were reported as regulating P metabolism in fish using flounder renal tubule cell cultures, these include stanniocalcin, rat prolactin, salmon/flounder somatolactin, bovine PTH (bovine), and salmon growth hormone (Lu et al. 1994).

Calcitonin (CT) has been discovered in humans as a hypocalcemic hormone produced by thyroid C cells more than 40 years ago (Copp et al. 1962; Foster et al. 1964). The mature CT molecule consists of 32 amino acids, which is released from an inactive precursor protein by proteolytic cleavage. The effects of CT are mediated through binding to a G-protein-coupled receptor - calcitonin receptor (CTR). CTR is present in several cell types, including in epithelial cells of the kidney neurons of the central nervous system, placental cells or lymphocytes. In mammals calcitonin acts on bone metabolism trough inhibition of bone resorption, since osteoclasts, unlike bone-forming osteoblasts, express high levels of the CTR (Huebner et al. 2008). In fish, however, the effect of calcitonin on plasma calcium levels is enigmatic and depends on the fish species and experimental conditions used (Shinozaki and Mugia 2002).

In teleosts calcitonin is produced in various tissues, but the ultimobranchial gland - UBG, is the main site of CT production (Sasayama 1999; Clark et al. 2002). The

ultimobranchial glands are considered the equivalent in teleosts for mammals C-cells located in the thyroid gland.

Stanniocalcin (STC, formerly called hypocalcin or teleocalcin) is a 50-kDa disulfide-linked homodimeric glycoprotein that was originally identified in fish and secreted from the corpuscles of Stannius (CS). The corpuscles of Stannius (CS) glands were identified in 1839 by Hermann Stannius (Stannius 1839). He described them as cream-colored bodies, or corpuscles, located ventrally on the surface of the fish kidney and he thought they were fish adrenals (Wagner and Dimattia 2006).

Stanniocalcin 1 (STC1) was first identified in fish as a hormone which inhibits calcium absorption in the gill and intestine and stimulates phosphate reabsorption in the kidney (Lafeber et al. 1988 ; Sundell et al. 1992). More recently a second member of the STC family, STC2, has been identified in fish (Luo et al. 2005) with only 30% identity to STC1 and current evidence suggests that it does not play a significant role in calcium regulation (Wagner and Dimattia 2006).

In mammals STC1 has a similar role as in fish and in the intestine inhibits calcium absorption and stimulates phosphate absorption and also stimulates phosphate reabsorption in the kidney and and increases Pi uptake in osteogenic cells, indicating that also in mammals STC1 plays an important role in calcium and phosphorus homeostasis (Wong et al. 1998; Yoshiko et al. 2003) STC1 has been shown to be upregulated by circulating vitamin D and also directly by blood calcium concentrations, while STC2 is controlled by a different mechanism including blood phosphate concentrations and klotho gene expression (Yahataa et al. 2003).

However common role STC play in fish and mammals there are differences in STC action between mammals and fish namely that mammalian STC1 does not regulate extracellular calcium levels as it does in fish (Gerritsen and Wagner 2005; Greenwood et al. 2009). Since the discovery of the mammalian homologs to fish STC there has been a progressively growing interest as to its possible role in humans and they have been identified as a novel markers for human cancer and of playing an important role in heart disease, transendothelial migration of inflammatory cells, embryo implantation and decidualization(Chen and Zhu 2008)

In the present study, an immunocytochemical and morphometric technique was used to study the adaptive responses of the calcitropic endocrine system to the availability of essential minerals, Ca and P by determining the distribution, number and size of the corpuscles of stannius and size and cellular activity of the ultimobranchial gland in rainbow trout larvae.

4.2 Materials and Methods

4.2.1 Imunohistochemistry

Localization of Stannius corpuscles and ultimobranchial gland was carried out by using respectively anti-fugu stanniocalcin 1:5000 (Schein et al. 2008), and anti-eel calcitonin 1:2000 (Bachem, UK). Larval sections were dewaxed, rehydrated through graded alcohols and tissues fixed in Bouin-Hollande fixative were immersed in a solution of 2% iodine in 70% alcohol followed by sodium thiosulphate 5% and rinsing in tap water and distilled water this step is performed in order to remove the mercury from the tissue, which could otherwise lead to unspecific staining. Since the method of revelation of the immunological reaction will be made with the utilization of peroxidase the endogenous peroxidase of the tissue has to be removed, to do that sections were incubated in PBS with 0.3% H_2O_2. Sections were incubated overnight at room temperature with the primary antiserum, and incubated for 45 min with the secondary antibody, anti-rabbit Ig whole molecule from rabbit peroxidase conjugate (Sigma-Aldrich, Spain) diluted 1:50. All antisera were diluted in PBS buffer pH 7.4 containing λ-carrageenan and 0.5% Triton X-100. Color development was carried out using Sigma *fast*-DAB (Sigma-Aldrich, Spain) as the chromagen. Sections were counterstained with Harris Hematoxylin, dehydrated, cleared in xylene and mounted with DPX (Sigma-Aldrich, Spain). Photographs were taken using Olympus BH-2 optical microscope coupled to an Olympus DP11 digital camera.

4.2.2 Morphometry

Stannius corpuscles

The initial approach taken to identify relative number and size of the CS was to make observations on several sections from each individual in a random sampling way. However it was observed that due to the lobular CS organization and their random distribution in the kidney, this approach was unsuitable, because what was perceived in one section has being a small CS could well be part of a larger one. So instead detailed analysis of CS in serial sections was carried out and this involved the measurement of

all the sections with positive STC 1 staining in order to accurately access the dimensions and number of CS.

In order to measure the secretary activity of the stannius corpuscles the number of corpuscles and volume were measured at D28 using Image J (freeware – NIH, http:\\www.rsbweb.nih.gov.ij). The measurements were made in all the sections obtained through the kidney that presented stanniocalcin staining. The volume was calculated by the formula

$$V = \sum (A * t)$$

Where *A* is the measured area and *t* the thickness of the paraffin section, the sum of this product for all the sections belonging to 1 corpuscle gave us the volume of each corpuscle. These individual volumes were also added to get the total gland volume in 1 individual.

Ultimobranquial gland

In order to measure the secretary activity of the ultimobranchial gland the area of nuclei and reference volumes of the glands were measured using Image J (freeware – NIH, http:\\www.rsbweb.nih.gov.ij) and calculated at D11 and D28 of the experiment. The reference volume was calculated by:

$$V = \sum_{1}^{4} A * t * n$$

Where *A* is the measured area of the UBG, *t* the thickness of the paraffin section and *n* the number of sections between measurements. The sum of this product gave us the reference volume of the UBG. After the gland was identified in the sections by each third slide was stained and the UBG measured. The 4 slides presenting the sections with the largest area were considered the middle ones and were taken into account for the reference volume. If some UBG did not span throughout the 4 slides then only 3 were taken into account for the volume calculation and the 4^{th} was considered 0.

For the nuclear area measurements the same slides used for the reference volume estimate were used. At least 25 nuclei from 5 or 6 individuals from each group were measured at D11 and D28 of the experiment.

4.2.3 Data analysis and Statistics

The groups were compared by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The statistical tests and graphs were made using GraphPadPrism v5 (GraphPad Inc., San Francisco, USA)

4.3 Results

4.3.1 Stannius Corpuscles

The morphology of the CS was similar to that previously described for salmonids. The CS are located in the dorsal part of the trunk kidney and are composed of an agglomeration of morphological units, a structure composed of a cluster of cells mostly arranged in a radial or globular fashion (Figure 4.1 A). The cells of the CS are rounded in shape, with a big nucleus and appear in clusters of variable sizes.

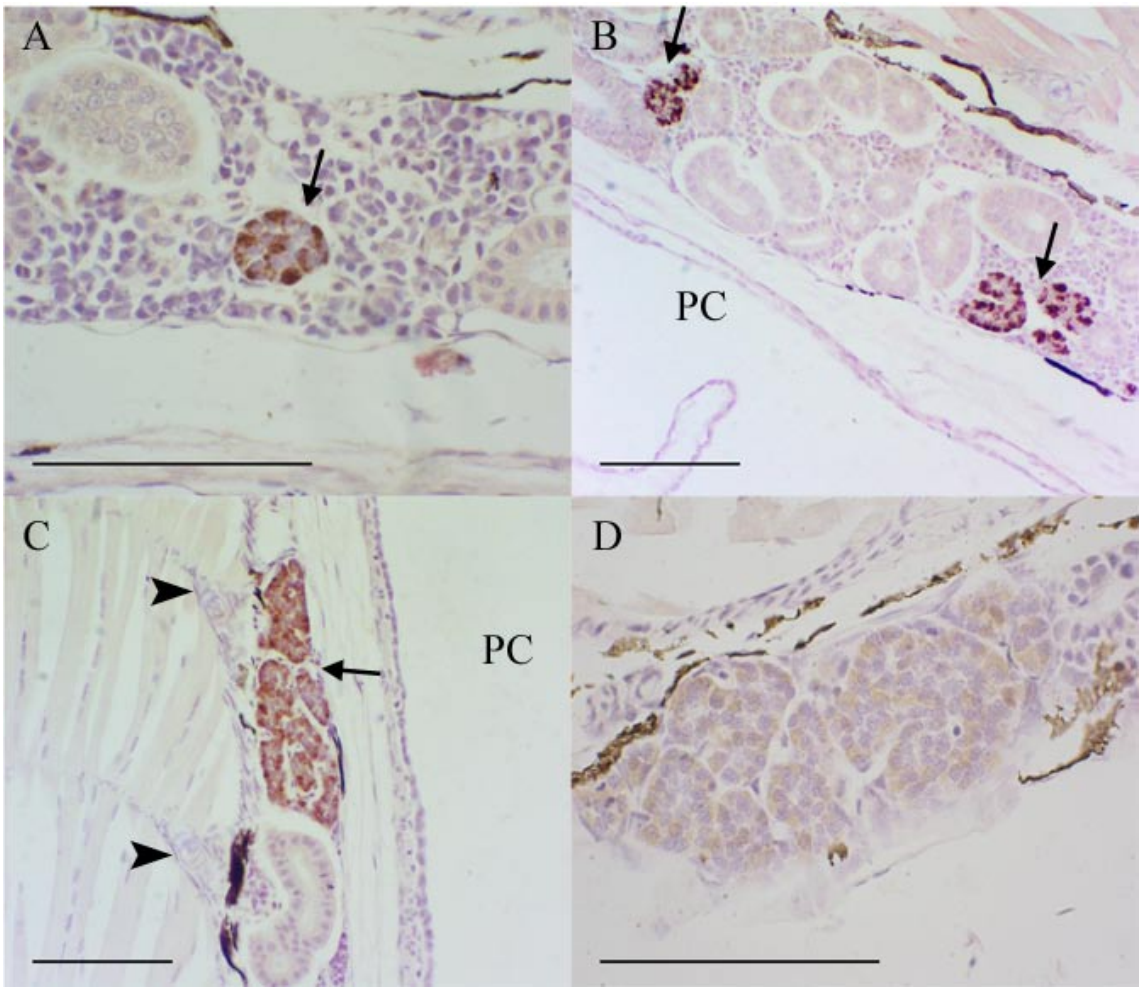

Figure 4.1 – Stannius corpuscles (arrows) morphology and distribution in the trout kidney demonstrated by staining with anti-fugu stanniocalcin sera. A- Morphological unit of the CS; B - The distribution of the CS in the kidney is variable with smaller corpuscles generally scattered throughout the kidney; C – The larger corpuscles are located in the anterior part of the trunk kidney, between the pleural ribs (arrow heads) and the peritoneal cavity (PC); D - Even in the largest corpuscles the globular arrangement of the morphological unit is visible. Scale bar 100 μm.

The distribution of the CS in the kidney is also variable (Figure 4.1 B), they are scattered through the trunk kidney and their exact position was observed to vary from individual to individual. Larger CS tend to be located in the mid anterior part of the kidney, they are sometimes located on the exterior surface of the kidney between the pleural ribs and the peritoneal membrane and no haematopoietic tissue (HT) or kidney tubules are visible (Fig 4.1 C). Smaller CS appear in greater number scattered in the HT in the mid posterior part of the kidney, in hematoxylin-eosin staining these clusters are almost indistinguishable from the HT, whilst the bigger CS are visible due to the particular pattern that the cell disposition creates (Fig 4.1 C, D).

The number and size of the corpuscles was evaluated in relation to the experimental conditions in 4 individuals per experimental group. The number of CS regions observed in one individual ranged from 3 to 13. The 4 individuals from group LP had 39 CS in total, group C had 17 CS and group HP and LCa had 22 CS and 20 CS, respectively. In Figure 4.2 it is clear that individuals in group 28LP possess both increased number and corpuscle volumes relative to the remaining experimental groups (Figure 4.2). In the LP group the four individuals had 7, 9, 10 and 13 corpuscles with a total volume of 0.013, 0.008, 0.013 and 0.010 mm^3, respectively. The number of corpuscles in the control group is more homogeneous and they have 3, 4, and 5 (n=2) corpuscles with 0.006, 0.009, 0.01 and 0.006 mm^3, respectively.

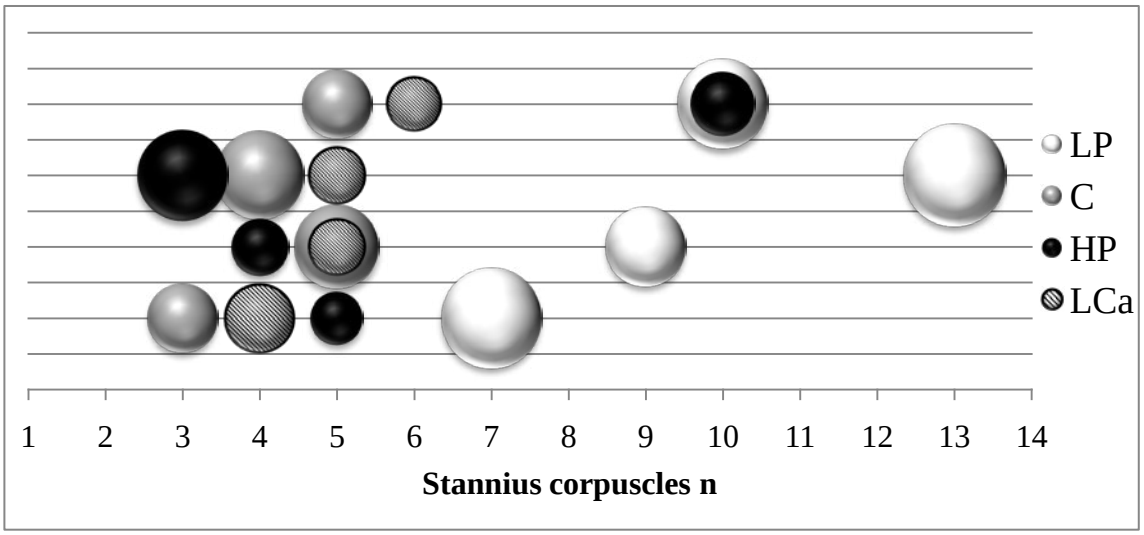

Figure 4. 2 - Number of Corpuscles of Stannius and their volume in the different individuals from each experimental group (n=4/group) at D28. Each sphere represents an individual; the x axis represents the number of corpuscles present in each individual and the size of the sphere corresponds to the sum of volumes of corpuscles measured for that particular individual.

Group LCa had a similar pattern to group C although the corpuscles are smaller and more homogeneous in size, its individuals had 4, 5 (n=2) and 6 corpuscles with a total volume of 0.006, 0.004, 0.004 and 0.004 mm^3. The HP group had the greatest intra-group variability with each of its subjects having 3, 4, 5 and 10 corpuscles with a total volume of 0.003, 0.004, 0.011, and 0.005 mm^3, it was also noticeable that although one of the individuals had 10 CS they were all quite small and their summed volume was similar to that of individuals in the same group with 3 and 4 CS.

The average volume of CS for the individuals in each experimental group is compared in Figure 4.3, where it is obvious that the amount of available P in the diet is inversely proportional to the CS volume, in groups LP, C and HP. The group with low levels of dietary calcium, LCa had an even lower CS volume than the other groups. With statistical differences between the LP and the HP ($p<0,05$) and LCa ($p<0,01$) groups

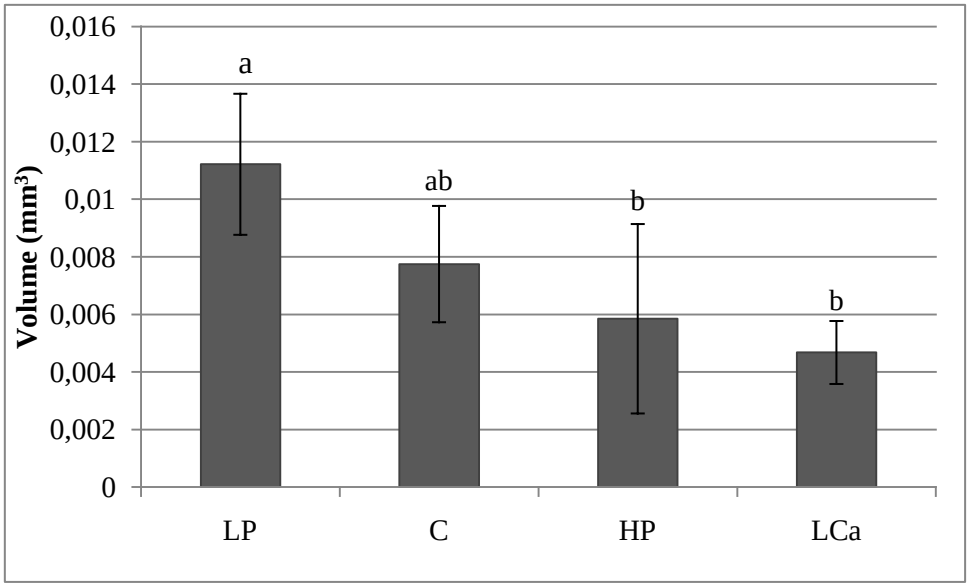

Figure 4.3 - Average volume of the Corpuscles of Stannius in each experimental group (n=4) at D28. Different letters represent statistical differences.

Analysis of the number of corpuscles in relation to the volume (Figure 4.4) suggests that variations in total CS volume in LP appears to be associated with an increase both in the number of corpuscles as an increase in the volume of pre-existing CS.

It is observed that individuals in group C have the majority of CS with volume between 0.001 and 0.005 mm^3 and assuming that this corresponds to the normal size pattern it is clear that the LP group have a greater number of CS for each size class and this is particularly striking in the increased number of smaller CS (Figure 4.4). The CS in group HP tend to be smaller in volume and of a more variable size than in group C,

whereas in group LCa the volume of CS are equally distributed between 0.0005-0.01 mm^3.

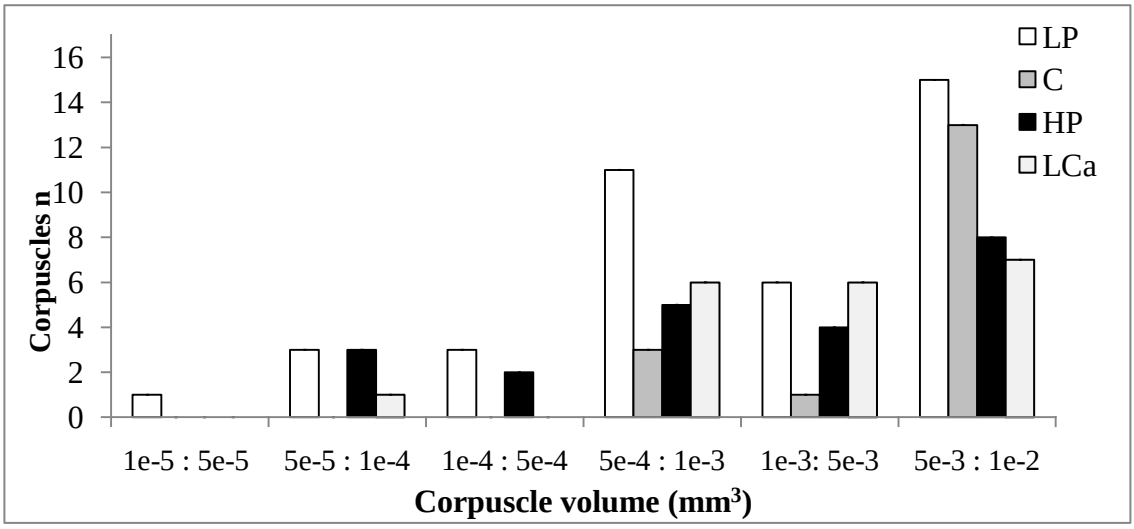

Figure 4.4 - Histogram representing the number of Corpuscles of Stannius in relation to their volume (mm^3) (n=4).

4.3.2 Ultimobranchial Gland

The ultimobranchial gland (UBG) of the trout is a paired gland which lies within the transverse septum, immediately beneath the circular musculature of the oesophagus, and caudal to the sinus venosus (Figure 4.6).

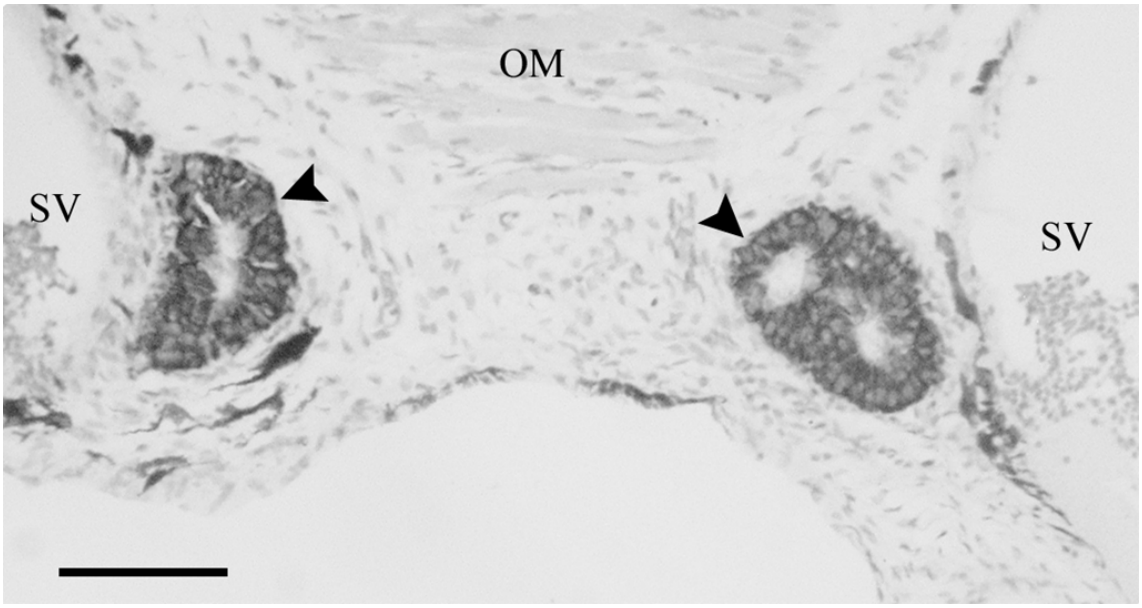

Figure 4. 6 – Ultimobranchial gland in a longitudinal section of 79 dpf rainbow trout, stained for calcitonin (CT) with a polyclonal antisera against eel CT. OM- oesophagus musculature; SV – Sinus venosus; Arrowhead – Ultimobranchial Gland. Scale bar 100 μm.

Each unit of the paired gland has a follicular structure with one or more central ductless cavities. The UBG is composed of a tall columnar epithelium; the cells are narrower on the cavity side giving them a "drop" appearance, with the nucleus usually occupying the upper third of the cell (Figure 4.2).

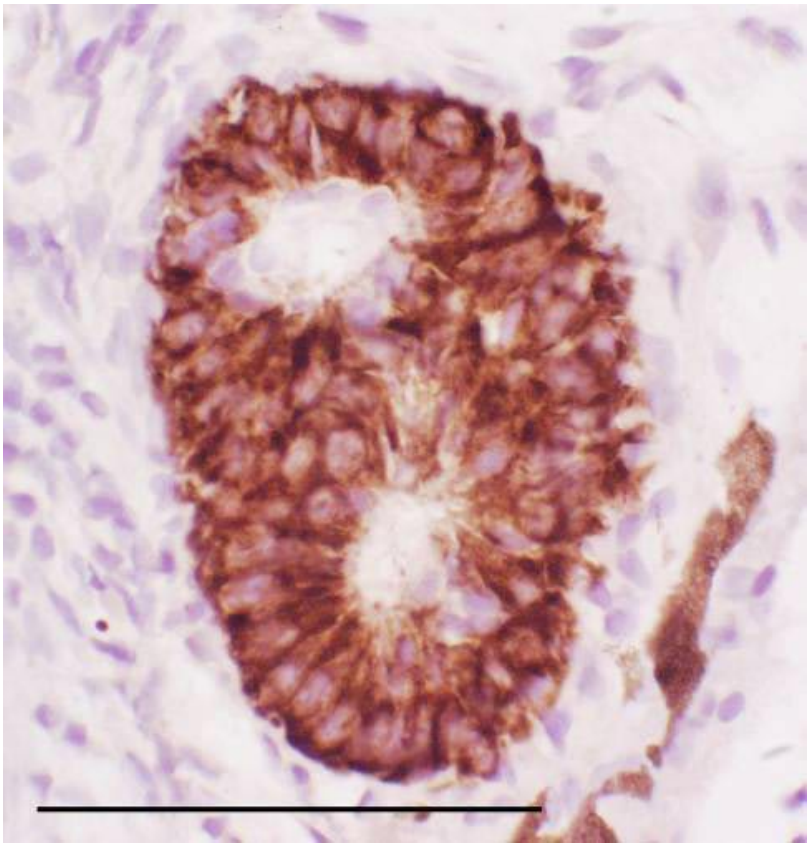

Figure 4.7– Detail of Figure 4.1.1 where the shape of the cells and nuclei are clearly visible. Scale bar 100 μm.

The nucleus of cells in the UBG is large and oval in shape and well distinguishable from the cytoplasm, which facilitates their measurement. The UBG volume was compared between fish maintained on different experimental treatments and at different time points during the experiment. As can be seen in Figure 4.8, the average reference volume measured at D11 varied from 0.0025 to 0.005 mm^3 whereas at D28 it varied between 0.0075 and 0.0085 mm^3. In the 15 days between D11 and D28 the UBG roughly doubles in size accompanying the fast growth of trout at this developmental stage. Neither the type of treatment nor their duration affects the volume of the gland in a significant manner.

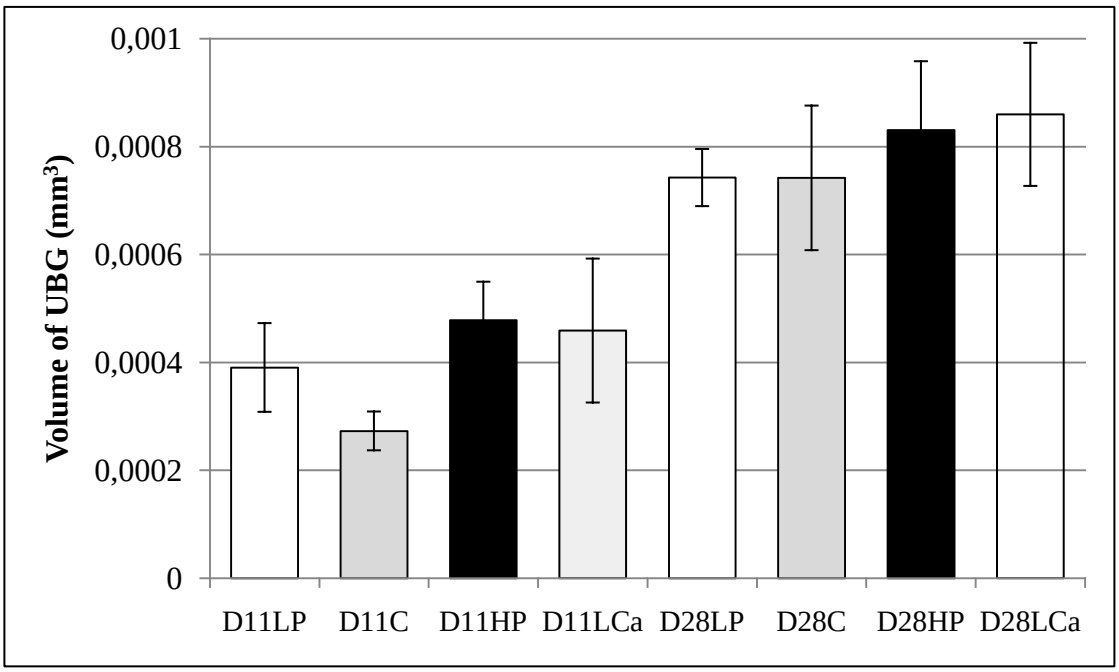

Figure 4.8 – Reference volume of UBG in 62dpf and 79 dpf raibow trout, with 11 and 28 days of experimental treatment respectively. (D11 - LP n=8; C n=7; HP and LCa n=9; D28 LP n=5; C and HP n=8 and LCa n=7)

The gland activity was also determined by measuring the nuclear area of the calcitonin producing cells. Figure 4.9 shows that the nuclear area of the UGB at D11 is significantly increased in trout from all the experimental treatments compared to the control;

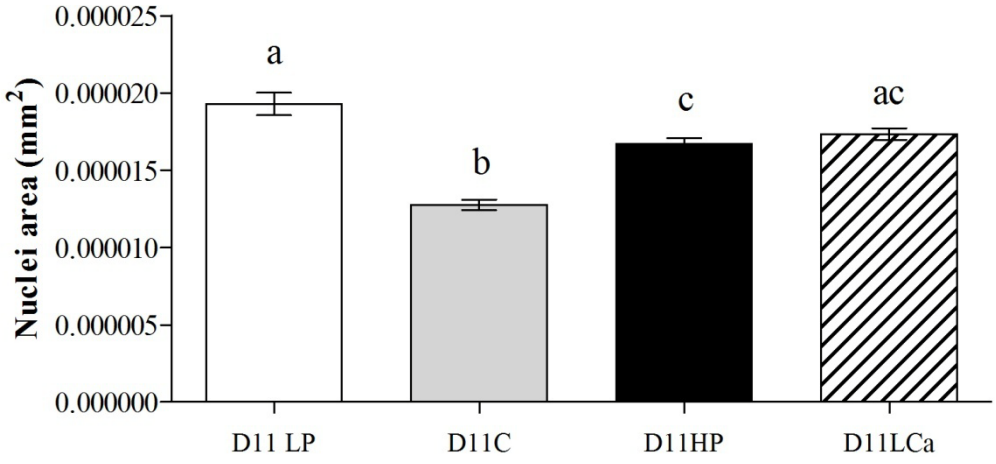

Figure 4.9 – Nuclear area of the UBG in 62dpf rainbow trout, with 11 days of experimental treatment. (LP n=92; C n=92; HP and LCa n=115 nuclei in 5 individuals) Different letters represent statistical differences.

this being 19 μm^2 for the LP group, 13 μm^2 for the C, and 17 μm^2 for HP and LCa groups. At this time point the low phosphorus group had an increased activity relative to trout fed a high P.

By day 28 of the experiment the nuclear area of trout on a low P diet, high P diet or control diet was not significantly different (Figure 4.10). The nuclear area of trout at D28 maintained on LCa was significantly lower than trout from D28LP and D28C.

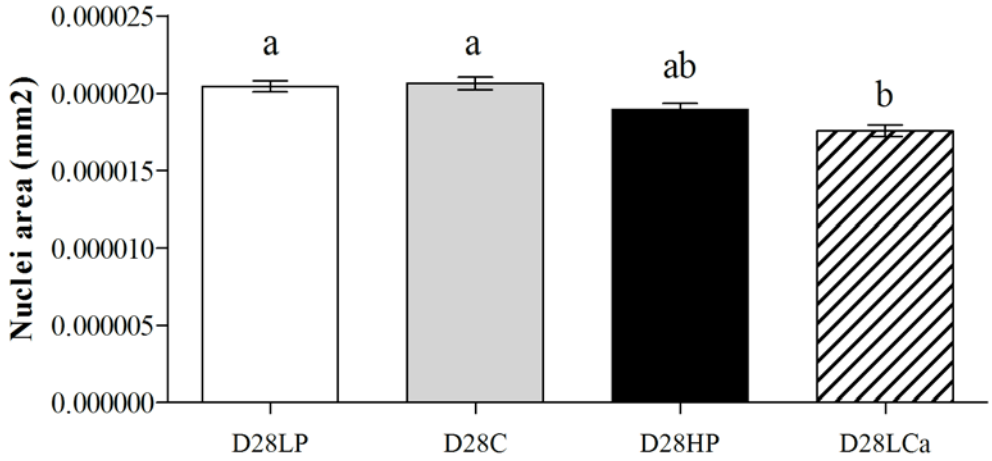

Figure 4.10 - Nuclear area of the UBG in 79dpf rainbow trout, with 28 days of experimental treatment (LP n=167; C n=121; HP n=151 and LCa n=148 nuclei in 6 individuals). Different letters represent statistical differences.

Nuclear area was evaluated within the same group at two different sample points in order to compare the effect of treatment duration (Figure 4.11.)

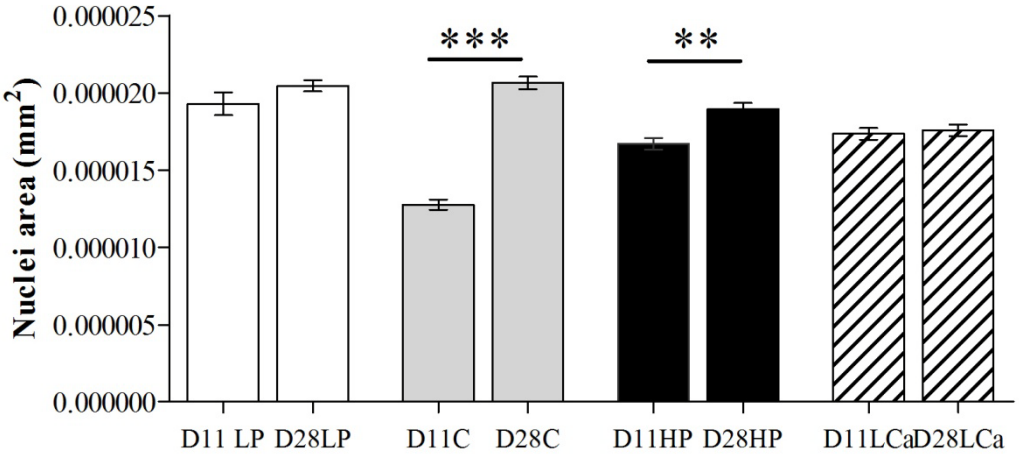

Figure 4.11 - Effect of the duration of experimental treatment on UBG nuclear area. (n=) *shows statistical differences with:**p<0.01 and ***p<0.001.

In the trout at D28 of the experiment the nuclear area was significantly bigger in control suggesting that UBG activity increases with somatic growth development, as did the reference volume. Activity increased also on group HP remained unchanged in trout maintained on diets with LP and LCa suggesting low Ca and high phosphorus diets for a long period reduce UBG activity. The experimental treatments did not cause histological modifications in the form or organization of the ultimobranchial gland.

4.4 Discussion

Although a lot of attention has been given in previous works to the ultrastructure of the CS cells, relatively little has been described about influence of experimental conditions on the distribution, number and size of the CS. This work aimed to identify stanniocalcin ir-cells in order to study the effect of dietary minerals on CS morphology. Previous experiments focused mostly on CS cell ultrastructure in relation to environmental factors such as, salinity, calcium or phosphorus concentration in the water.

Salmonids have been reported to possess 4-6 corpuscles (Krishnamurthy and Bern 1969; Matty 1985). This number is corroborated by the present work as seen in the C, LCa and HP groups (this last one had an outlier individual with 10 CS), but in the case of LP all the individual had more than 7 CS which supports the notion that these animals are producing higher amounts of STC 1 (chapter 5). The increased number of small CS observed in D28LP, in relation to C may indicate recruitment of individual progenitor CS cells to form new clusters. This phenomenon has previously been described during embryonic development of chum salmon (Kaneko et al. 1992) where it was demonstrated that CS arise from individual cells, which first appear among the epithelial cells of the embryonic nephric ducts. These individual CS cells already contain stanniocalcin prior to their organization into typical CS tissue, as proven by immunohistochemical findings and also by the fact that after stanniectomy fishes have the ability to an upregulate STC production in the kidney and re-establish normal calcium balance (Wagner and Dimattia 2006). In the present work cell recruitment, indicated by isolated cells which are STC positive, was not observed possibly because of the developmental stage studied and timing of experimental sampling.

Adult teleost, including salmonid CS glands possess 2 cell types, morphologically distinct - 1 and 2. Both contain STC positive secretary granules when immunogold staining and visualization by electron-microscopy is carried out. The presence of two cell types in the CS has been proposed to reflect different physiological conditions of a single cell type, rather than functionally different cell types (Bonga et al. 1989). This is supported by the results of the present study in which all cells were STC positive and had a similar morphology at the light microscopy level.

STC transcripts are widely expressed in the fish body including kidney, ovary and testis, although the CS glands have by far the highest level of gene expression (over 100-fold greater than any other tissue) (McCudden et al. 2001). Recent work (unpublished – Canario AVM) has revealed the presence of duplicated STC1 and STC2 paralogue genes in fish that arose from genome duplication events, STC1A, STC1B, STC2A, and STC2B. Polyclonal antibodies against synthetic peptides specific to amino acids in the C-terminal regions of STC1, STC1B and STC2A proteins have been produced. The tissue distribution of these peptides was analysed by immunohistochemistry in several teleosts, including the rainbow trout and it was evident that STC1A protein was produced exclusively in the CS (Schein et al. 2008). So although the several members of the stanniocalcin family are widely expressed, presumably the increase in activity of in the CS gland in the present study must reflect an increased production of STC1A only.

The CS glands of euryhaline fish kept in calcium-deficient sea water appear to be inactive but become activated when fish are transferred to seawater, reflecting the 10-100 fold increase in the concentration of calcium in seawater (Bonga 1980; Bonga et al. 1989; Kaneko et al. 1992). Such observations have been taken to suggest that CS activity is directly related to environmental calcium concentrations. Similar results were obtained in the present work as trout maintained on low calcium diets (LCa) had a lower index of CS activity in comparison to trout maintained on diets with normal Ca levels. The mechanism by which STC modifies calcium uptake at the fish gill remain to be established but has been proposed to result from the regulation of apical membrane calcium channels in the gill or by redirecting blood flow away from the gill (Tseng et al. 2008)

The involvement of STC in phosphorus metabolism is still not completely clarified, it is known from previous work that in kidney it stimulates phosphate reabsorption in order to chelate excess $Ca2^{+}$ in the serum (Wagner et al. 1997a). The present work demonstrates clearly that chronic exposure of trout larvae to dietary phosphorus restriction significantly increases the activity of CS and that exposure to high doses of dietary phosphorus has the exact opposite effect, and is similar to the effect observed when Ca is restricted.

While in mammals, high-affinity STC-1 receptors/ binding proteins have been described in a number of different tissues, nothing is known about receptor structure in mammals or fish. The regulation of STC-1 production is still not well known (Wagner and Dimattia 2006), but appears in fish to involve CaSR (calcium sensing receptor). CaSR is highly expressed in fish CS cells at levels comparable to its expression in mammalian parathyroid gland cells where it is known to regulate parathyroid hormone production, providing evidence for calcium-stimulated STC-1 release in fish via CaSR activation (Greenwood et al. 2009). CaSR is suggested to represents a key consistent component of the major calcium regulatory systems in fish and albeit hypocalcemic (STC-1) mechanisms dominate in fish, whereas hypercalcemic (PTH) systems predominate in mammals. This gives extreme importance to the study of mineral regulatory mechanisms in fish.

The ultimobranchial gland is the major site of calcitonin1A production in fish (Clark et al. 2002). Since the discovery of calcitonin in fish, establishing a clear cut role for the hormone in calcium homeostasis has proven to be exceedingly difficult because there are poor correlations between plasma calcium and CT levels. In fish CT cells are also much less responsive to changes in extracellular calcium levels than their mammalian counterparts (Wagner et al. 1997b). To our knowledge there is only 1 previous study that refers to the effect of mineral challenge on the UBG activity, it describes a decreased activity of UBG cells under low calcium water in stickleback (Bonga 1980). Most of the studies which exist are in vitro or are based on the administration of calcitonin to determine its action and has led to discrepant results. Chan *et al.* (1968) found that the hypocalcemic effect of CT in freshwater eels was eliminated when the fishes underwent stannioectomy, implying that STC was responsible for mediating the calcitonin effect.

It has been reported that the rainbow trout do not express calcitonin in the gills (Fouchereau-Peron et al. 1990; Hidaka et al. 2004), probably due to the fact that they are fresh water fish, which decreases the importance of this hormone in this fish as a direct regulator of calcium in the gill. The theory which currently has most support is that calcitonin functions as a hypocalcaemic hormone in fish by stimulating STC secretion which in turn acts through GCAT (gill calcium transporter), decreasing the blood flow that goes through them (Wagner et al. 1997b) and so decreasing the uptake of calcium from the environment. Nonetheless, other studies have convincingly demonstrated that CT can raise calcium levels in fish (Glowacki et al. 1985; Fouchereau-Peron et al. 1987; Oughterson et al. 1995).

A detailed description of the development of the UBG and calcitonin expression in the rainbow trout is not the aim of this study as it has previously been described (Robertson 1969; McMillan et al. 1976 ; Hooker et al. 1979; Hidaka et al. 2004). UBG is detected as 2 disk like structures at 17 dpf in trout and calcitonin immunoreactivity is detected in them at 19dpf, around 2 days before hatching (Hidaka et al. 2004). Calcitonin1 mRNA is also present in the cytoplasm of the ova of several fish (Buznikov 1984 ; Clark et al. 2002) which suggests it is important on the initial stages of larval development, when the UBG has not yet developed or functional. In the present study the reference volume and nuclear area of the UBG of 62 and 79dpf rainbow trout under dietary mineral restriction/excess was analyzed. By this stage the appearance of the UBG was similar to the described in adult fish (Robertson 1969; McMillan et al. 1976).

The measured UBG reference volume was not affected by the dietary treatments even when nuclear activity was altered, this might be because the normal development of the gland masked the effect of the treatments or simply because the increased nuclear volume did not modify the overall gland size.

Regarding the variations observed in nuclear activity, if the control trout (C) group is considered as having the normal pattern of UBG development, it is clear that at D11 all the experimental groups had a higher nuclear activity, indicative of a more active gland. Again the conflicting effect of calcitonin on calcium balance comes to light and in the present study, while the higher activity of the UBG in the LP group could be justified by

the described hypocalcemic role of calcitonin, high phosphorus and low calcium also appear to stimulate the activity of the UBG suggesting calcitonin may behave as a hypercalcemic factor in these animals.

At D28 the possibility that the observed calcitonin effects are mediated in one way or another by STC should not discarded (Wagner et al. 1997b). This would be in accordance with the results obtained for D28HP and D28LCa groups which have decreased UBG activity and presented also a smaller CS volume suggesting that both this glands presented reduced activity. Maintaining low levels of these hormones may facilitate calcium transport from the gills.

No relationship has been established until now about the effect of calcitonin on phosphorus metabolism in fish or vice-versa, although in mammals it has an effect (Sugiura and Ferraris 2004a). In this experiment the variation in minerals in being made by the ingestion route, there is no evidence of the endocrine action of calcitonin in the gastrointestinal tract of fish, and while its action at the level of the gill maybe related to STC no other indication exists of other functions, that could justify the contradictory results obtained. In fugu calcitonin as been shown to also be expressed in brain, spinal cord, gill, gut, eye, liver, heart, kidney, spleen, and gonads (Clark et al. 2002) suggesting a role as a neuropeptide, have local controlling actions on the functions of kidney cells, where in mammals it may have both hypo and hypercalciuric actions (Costanzo 1992).

4.5 Conclusion

From this experiment it can be concluded that CS are enlarged under dietary phosphorus restriction and reduced in volume under dietary calcium restriction confirming the mineral status of the fish and the hypocalceamic role of stanniocalcin.

The measured activity of the ultimobranchial gland gave contradictory results showing that reference volume was not affected by dietary calcium or phosphorus but the nuclear activity varied with duration and type of dietary treatment, with no conclusive results about the role of calcitonin.

PART 5

MOLECULAR RESPONSE OF TROUT (*Oncorhynchus mykiss*) FRY TO DIETARY PHOSPHORUS AND CALCIUM LEVELS

5.1. Introduction

Recent studies have identified a series of genes that are phosphorus responsive in the intestine, pyloric ceaca and kidney of adult rainbow trout, by subtractive hybridization RT-QPCR and microarray (Sugiura and Ferraris 2004a; Kirchner et al. 2007; Sugiura et al. 2007). A number of these genes are modified only 2 days after the beginning of the experimental treatment and include inorganic phosphate transporters, meprin A1, vitamin D receptor and S100. This could indicate that nutrient responsive genes can identify important metabolic pathways and be used as early markers of nutritional status replacing the usual techniques that are not very specific and require the presence of clinical signs of deficiency, such as reduced growth, mineral content, deformities etc (Rodehutscord 1996; Kirchner et al. 2007). No studies of phosphate responsive genes exist during ontogeny of teleost fish and it remains to be established if manipulation of dietary phosphate has effects similar to what is seen in adult teleosts.

In mammals, phosphorus uptake and homeostasis is accomplished by regulation of inorganic phosphate (Pi) transporters in epithelial cells of the intestine and kidney. Type 2 NaPi transporters (NaPi-II) are the best-known P-responsive gene and are responsible for regulated epithelial Na-dependent Pi transport in all vertebrates. They mediate transcellular Pi transport from the intestinal and renal tubular lumen across the apical membrane of the epithelial cells (Werner and Kinne 2001). Mitochondrial Pi carrier (MtPi) protein was induced threefold in adult rainbow trout by a low-P diet (Sugiura and Ferraris 2004a). This protein transports Pi from the cellular cytosol to the mitochondria for use in oxidative phosphorylation and may be a vital compensatory response to P deprivation, which is known to decrease ATP production. (Sugiura, Ferraris 2004)

One of the genes seen to be differentially expressed in adult rainbow trout fed LP diet (Sugiura and Ferraris 2004a) was meprin A1 which is a brush-border metalloendopeptidase capable of hydrolyzing a variety of peptide and protein substrates. Meprins are members of the 'astacin family' of metalloendopeptidases and are capable of hydrolyzing a variety of peptide and protein substrates. Two isoforms have been isolated from the kidney of mouse, designated A (contains both alfa and beta subunits) and B (contains only the beta subunit). In mammals both meprin subunits cleave a wide

range of proteins and biologically active peptides, although they differ considerably in their cleavage specificity (Kruse 2003). They can process compounds of the extracellular matrix such as laminin-V, collagen-IV, fibronectin and nidogen 1, as well as cytokines and peptide hormones, including bradykinin, angiotensins and gastrin (Yamaguchi 1992; Bertenshaw et al. 2001; Kruse 2003), melanocyte-stimulating hormone, neurotensin, luteinizing hormone releasing hormone, transforming growth factor-alpha, and parathyroid hormone (McKusick and 1997). In zebrafish meprins are expressed with variable abundance in kidney, liver, intestine, epidermis, brain, heart and gills, and contrary to humans and mice are encoded by three genes, corresponding to two homologous A subunits (1 and 2) and one B subunit. The zebrafish meprins share many similarities with their mammalian homologues but have specific properties that probably affect secretion and cleavage specificity. Taken together, these findings suggest meprins contribute to epithelial differentiation, matrix remodeling and cell migration, as well as to inflammatory processes, tumour growth and metastasis (Schutte et al. 2007).

The vitamin D endocrine system plays a vital role in mineral homeostasis, endocrine, immune, and neural function (Norman 2006). The active metabolite of vitamin D_3, $1\alpha,25(OH)_2D_3$ is produced in the kidney and liver, binds to its receptor, the vitamin D receptor (VDR), with high affinity and subsequently regulates the expression of several genes. The hormone–receptor complex induces calcemic and phosphatemic effects that result in normal bone mineralization and remodeling (Jurutka et al. 2007). The crucial role of VDR in mediating the function of $1\alpha,25(OH)_2D_3$ is shown by the occurrence of disordered calcium and phosphorous homeostasis in humans and animal models that have mutations of the VDR (Kato 2000). VDR not only mediates the action of $11\alpha,25(OH)_2D_3$ in calcium/phosphate translocating tissues, primarily intestine, but also elicits a myriad of apparent bioactivities in other major cell systems in mammalian organisms, including immune, neural, epithelial, and endocrine (Ferrari et al. 1998). Immunohistochemistry of VDR reveals it is widely expressed in tissues of the adult zebrafish, primarily in epithelial cells of gills, tubular cells of the kidney, and absorptive cells in the intestine but it is also expressed in endocrine and neural tissues (Craig et al. 2008). In fish VDR transcripts were found to be transiently modified soon after mineral challenge of adult rainbow trout and injections of vitamin D caused hypercalcemia in tilapia (Srivastav et al. 1998; Sugiura and Ferraris 2004a).

Another of the differentially expressed proteins in P deficient adult rainbow trout was S100 11A also denominated S100C, This protein belongs to a gene family containing 25 members and are the largest subgroup of the EF-hand Ca^{2+}binding protein family in humans. Although the S100 proteins are expressed in many tissues, specific proteins tend to be expressed in one particular tissue or cell type. S100 proteins regulate processes such as cell growth and motility, cell cycle regulation, transcription and differentiation (Heizmann et al. 2002; Donato 2003). This protein is thought to be involved in the calcium dependent regulation of actin filaments with consequent regulation of actomyosin ATPase and interaction with annexin A1 through calcium-dependent membrane aggregation, important for cell vesiculation and membrane cross linking (Bianchi 2003; Santamaria-Kisiel et al. 2006). Nuclear translocation of S100A11 causes diminished cell proliferation, suggesting the protein might be involved in the contact inhibition of cell growth (Donato 2003).

An important component of the skeleton is the extracellular matrix particularly in larvae and juveniles where it is actively developing. The effect of dietary minerals on the genetic expression of extracellular matrix proteins in fish is not well described and in the present study change in transcript abundance of osteonectin in response to modified mineral availability was analysed. The extracellular matrix (ECM) of skeletal tissues provides a structural framework for mineralization and modulates functions such as cell adhesion/migration and growth factor signaling and in this way contributes to maintain tissue homeostasis. The organic extracellular matrix is composed of a series of nonhomologous, function-based regulatory factors that include osteocalcin, osteonectin, osteopontin and bone sialo protein (Gericke et al. 2005; Hall 2005; Redruello et al. 2005b; Cohen 2006).

Osteonectin is a multifunctional metal-binding glycoprotein encoded by a single-copy gene that displays a high degree of interspecies sequence conservation. OSN expression has been used as a marker of the osteoblastic response since its first description as a linker between mineral and collagen in human bone. OSN is implicated in mineralization of bone and cartilage. High concentrations of calcium ions increase expression of OSN and also other markers of terminal differentiation, followed by an increased production of mineral matrix (Termine et al. 1981; Redruello et al. 2005b).

Osteopontin or Secreted phosphoprotein 1 (SPP1), is a secreted phosphoglycoprotein that was first identified in bone extracellular matrix but is expressed by various cell types, including osteoclasts, osteoblasts, chondrocytes, macrophages, activated T cells, smooth muscle cells and epithelial cells, and is present in several other tissues including kidney, placenta, smooth muscle and secretary epithelia. Osteopontin may be an important factor in bone remodeling, since it plays a role in anchoring osteoclasts to the mineral matrix of bones and promotes chemotaxis during bone resorption. It also accumulates in mineralized bone matrix during endochondral and intramembranous bone formation, enhances osteoblastic differentiation and proliferation, and increases ALP activity (Choi et al. 2008).

The objective of this work was to test the effect of dietary mineral content on trout fingerlings by evaluating the expression of genes known to be modified in adult trout and relate these findings with the expression of genes involved in bone formation and development and also with the expression of the mineral regulating endocrine factors, stanniocalcin and calcitonin.

5.2 Methods

5.2.1 Gene expression

The method chosen to analyse modifications in gene expression in response to dietary P and Ca was RT-QPCR using SYBR green chemistry. Three different groups of genes were analysed, those described in previous work as having a modified expression in the intestine after dietary P restriction. These genes included, intestinal phosphorus/sodium co-transporter (INaPiIIb), mitochondrial inorganic phosphate carrier (MtPi), vitamin D receptor (VDR), meprin 1A (MEP) and S100 calcium binding protein (S100) (Sugiura and Ferraris 2004a; Kirchner et al. 2007; Sugiura et al. 2007). Another group of genes identified as important in bone formation were also analysed and included osteonectin (OSN) and osteopontin (OSP). Endocrine hormones known to be important in mineral balance were also analysed, calcitonin (CT) and stanniocalcin (STC). Two other genes evaluated, 18S and β-actin, were tested for normalization of the RT-QPCR analysis.

In PCR any nucleic acid sequence present in a complex sample can be amplified in a cyclic process to generate a large number of identical copies that can readily be analyzed. The main drawback of the original PCR method was that quantification was exceedingly difficult as after the initial logarithmic phase of amplification PCR gives rise to essentially the same amount of product independent of the initial amount of DNA template and the kinetics of PCR reaction may be very variable. This limitation was resolved in 1992 by the development of real-time PCR by Higuchi et al. (Higuchi 1992; Kubista et al. 2006). In real-time PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into the reaction and are proportional to the amount of product formed. The number of amplification cycles required to obtain a particular amount of DNA molecules is registered and assuming amplification efficiency which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules initially present in the sample (Kubista et al. 2006).

When quantifying mRNA, real-time PCR can be performed as either a one-step reaction (Q-RT-PCR), where the entire reaction from cDNA synthesis to PCR amplification is

performed in a single tube, or as a two-step reaction (RT-QPCR), where reverse transcription and PCR amplification occur separately. While one-step real-time PCR is thought to minimize experimental variation because both enzymatic reactions occur in a single tube, it uses RNA as starting template, which is prone to rapid degradation, mainly if same sample is assayed on several occasions over a period of time. One-step protocols are also reportedly less sensitive than two-step protocols (Battaglia 1998; Wong and Medrano 2005). Two-step real-time PCR separates the reverse transcription reaction from the real-time PCR assay, allowing several different real-time PCR assays on dilutions of a single cDNA. Because the process of reverse transcription is notorious for its highly variable reaction efficiency, using dilutions from the same cDNA template ensures that all reactions in different assays have the same amount and quality of template, this makes data from two-step real-time PCR quite reproducible (14). The disadvantage of this method is that it presents increased opportunities of DNA contamination in real-time PCR (Wong and Medrano 2005). There are several fluorescent reporters that can be used in Q-PCR: 1) labeled primers and probes (eg. Taqman and Molecular Beacons) which are sequence specific but also more expensive and are most useful in multiplex assays where several products are amplified in the same tube and detected in parallel; and 2) DNA binding dyes (ex. asymmetric cyanines like SYBRgreen and BEBO), which emit fluorescence when bound to dsDNA. This technique is very flexible and is cheaper because one dye can be used for different gene assays. The main pitfall of this strategy is that dyes can bind to any double stranded DNA including undesired primer–dimer products and good quality control is essential and based on validation of the assays by melting curve analysis. As long as a single target is detected per sample there is not much of a difference in using a dye or a probe (Wong and Medrano 2005; Kubista et al. 2006).

5.2.2 Total RNA Isolation

Total RNA was extracted from 5 individuals were extracted with TriReagent (Sigma-Aldrich, Madrid, Spain). This reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution which effectively dissolves DNA, RNA, and protein on homogenization of tissue sample. Due to the size of the fishes from D28, fishes were weighed sectioned transversely, at the level of the pectoral fin; each half was extracted separately, and put in 2ml microcentrifuge tube with 1 ml of TriReagent being added/100 mg fish. The tissues were homogenised using an Ultra-Turrax® T25 Basic

homogenizer (IKA, VWR, Carnaxide Portugal), placed on ice and then centrifuged for 10 minutes at 4ºC and 12000rpm and the supernatant containing RNA and protein transferred to a new microcentrifuge tube. Subsequently 200 µl of chloroform (Merck, VWR, Portugal) was added per ml of Tri reagent used, vortexed for 15 seconds and incubated at room temperature for 10 minutes, followed by centrifugation for 15 minutes at 4ºC and 12000 rpm. This step separates the mixture into 3 phases: a colourless upper aqueous phase containing the RNA, the interphase containing DNA, and a red organic phase containing proteins. The upper aqueous phase was transferred to a new microcentrifuge tube and 500 µl of isopropanol added/ml Tri reagent. The RNA was allowed to precipitate by vortexing and incubating at room temperature for 10 minutes and collected by centrifuging for 10 minutes at 4ºC and 12000 rpm. The supernatant was discarded and the pellet was washed with 1 ml of EtOH 75%/ ml Tri reagent followed by centrifugation for 5 minutes at 4ºC and 7500 rpm this procedure was repeated 3 times. The pellet was the dried over ice and ressuspended in 100 µL of sterile miliQ water, after complete dissolution of the pellet the RNA extracted from the 2 half's of each individual were mixed.

The RNA extracted was quantified using a *spectrophotometer* (GeneQuant, Amersham Bioscience, Lisbon, *Portugal*) and the concentration determined and the purity assessed using the ratio 260/280. RNA quality was classified as adequate when the ratio was >1.7. The quality of extracted total RNA was determined by agarose gel (2%) electrophoresis using ethidium bromide. Two predominant bands of small (2 kb) and large (5 kb) ribosomal RNA, low molecular mass (0.1–0.3 kb) RNA, and discrete bands of high molecular mass (7–15 kb) RNA were observed when agarose gels were examined in UV light. Samples displaying an appropriate pattern of bands after electrophoresis were aliquoted and stored at 80ºC until required.

5.2.3 RNA purification

In order to remove contaminating genomic DNA from the samples 4 µg of total RNA was treated with DNase using Ambion DNA-*free*™ (Bioportugal, Lisboa, Portugal). In brief, 4 µg of total RNA was mixed with 0.1x volume of DNase I buffer and 1 µl of rDNAseI (2U/µl). This mixture was incubated at 37ºC for 30 minutes, and the DNase inactivated by addition of 0.1x volume of inactivation reagent for 2 minutes at room temperature. The solution was then centrifuged at 13000 rpm for 1.5 minutes and the

supernatant containing the DNA free-RNA was transferred to a new tube and stored at -20ºC until further use. The purified RNA was quantified using Quant-iT™ RNA BR Assay Kit (Invitrogen, Barcelona, Spain) in which a fluorophore (in this case RiboGreen) that has high affinity for RNA binds to it and fluoresces. Total RNA (1ul) was diluted in (1:200) working solution, vortexed 2-3 seconds and incubated at room temperature for 2 minutes. The fluorescence at 260 nm was read using a Qubit ™Quantiation Platform (Invitrogen) which gives the concentration of RNA present in the sample.

5.2.4 cDNA synthesis

As mentioned earlier RNA is difficult to work with because it is prone to degradation by RNases that occur naturally in the environment. To prevent this, samples are reverse-transcribed into a more stable DNA form. The product of this reaction is called complementary DNA (cDNA) because its sequence is the complement of the original RNA sequence. A problem with cDNA production is that not all RNA's are reverse-transcribed with the same efficiency, one way to overcome this problem to ensure a representative sample of the RNA population is to prime reverse transcription from random starting positions on the RNA. DNase treated total RNA was used for first strand cDNA synthesis which was carried out in final volume of 20µL. The RNA was added to milliQ sterile water and warmed for 10 minutes at 65ºC to ensure loss of secondary structures that could interfere with the annealing step. This solution was the added to a mixture containing, 0.05 µg of Random Hexamer Primer, p(dN)6, 0.25 mM dNTP mix, 20 U of Recombinant RNasin® Ribonuclease Inhibitor, RT buffer (5 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, 10 mM DTT) and 10U MMLV-RT, all reagents from Promega (VWR, Carnaxide, Portugal). The RT reaction took place in a multigene thermocycler (Labnet, NJ, US) programed for 10 minutes at 25ºC followed by 50 minutes at 42ºC and final heating for 2 minutes at 70ºC to inactivate MMLV-RT. Forty individual cDNA synthesis reactions corresponding to five individual animals per stage and treatment were performed. Two random samples were selected as negative controls, in these reactions RNA was omitted from the cDNA synthesis; these reactions served as controls for the amplification of genomic DNA and are named –RT controls. To assess the success and quality of the cDNA synthesis, PCR amplification of 18s rRNA was carried out with all samples in a 25µl reaction containing 1 µl of cDNA (diluted 1/10),

1.5 mM $MgCl_2$, 0.1 mM dNTP's, 1 pmol/µl of forward, 0.025U of Eurotaq *Taq* polymerase (Euroclone, Italy) (5'-TCAAGAACGAAAGTCGGAGG-3') and reverse primer (5'-GGACATCTAAGGGCATCACA-3'). The reaction was performed in a Multigene thermocycler (Labnet, NJ, US) and the program used was: 3 minutes at 95ºC followed by 21 cycles of 30 seconds at 95ºC, 30 s at 56ºC and 30 seconds at 72ºC, followed by a final step of 3 minutes at 72ºC. The reaction products were fractionated on 2% agarose gels stained with ethidium bromide and reaction products evaluated.

5.2.5 Cloning of genes of interest

Database search

The quantification method used with the RT-QPCR method was the relative standard curve method and required a standard. For this reason target genes of interest were cloned and to do this their nucleic acid sequence were extracted from the NCBI database (http://www.ncbi.nlm.nih.gov/) or the trout EST contig database (http://public-contigbrowser.sigenae.org:9090/Oncorhynchus_mykiss/index.html) and used for as templates for PCR primer design. The database and corresponding accession number of each gene is described in table 5.1.

Table 5.1- Name of the gene, accession number and database where it is localized.

Gene	Database	Accession number
Calcitonin	Genbank	AB094135.1
I NaPi2b	Genbank	AF297184.1
Meprin 1A	Sigenae	BX084554.3
MtPi	Genbank	AAH46007
Osteonectin	Genbank	U25721.1
Osteopontin	Genbank	AF204760.1
S100CaBP	Genbank	EE605180
Stanniocalcin	Genbank	AF326318.1
Vit D receptor	Genbank	AY526906.1

Based on the published sequences, the putative genomic organisation of the target trout genes was established *in silico* using the zebrafish (*Danio rerio*) genome database (http://www.ensembl.org/index.html). The zebrafish scaffolds giving the most significant hit by tBLASTx analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Translations&PROGRAM=tblastx&BL

AST_PROGRAMS=tblastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) (Altschul et al. 1990) was recovered, and using the pairwise alignment tool Spidey mRNA-to genome (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) (Wheelan et al. 2001) it was possible to identify putative exon/intron boundaries (Table 5.2). This information was used to design primers localised in different exons or exon/intron boundaries for amplification of the target amplicon of interest. Primers for gene cloning were designed in Primer 3 software (http://frodo.wi.mit.edu/) (Skaletsky 2000).

Table 5.2 – Primer name, primer sequence and putative genomic localization of the primers of the candidate genes.

Primer name	Primer sequence	Primer localization	
NS_CT.For	AGAGGAACTGGAGCAACAAG	Exon	1
NS_CT.Rev	AGAAAAAGAGAGCAGGGACA	Exon	3
NS_INaPi.For	CAGTGCCATCATAGAGCTGGATA	Exon	1/2
NS_INaPi.Rev	AGGCAGGTTAGCATAGGCAAAG	Exon	5
NS_Meprin1A.For	AGCTAGATGGGTGCTGGTCTATG	Exon	7
NS_Meprin1A.Rev	TCGTAGTCGTATGGCGTGTTCTG	Exon	8
NS_mtPi.For	TGCTTGGAGAGGAGAACACCTAC	Exon	5
NS_mtPi.Rev	ACCCTTGTAGAACGCCCATACTC	Exon	5
NS_OSN.For	GCATTCACCGCTTCCATTAC	Exon	5
NS_OSN.Rev	TCCACCTCACACACCTTTCC	Exon	6
NS_OSP.For	AGAGCCCAGAGAGCCAGAA	Exon	8
NS_OSP.Rev	CAGCAGGATGAGAGAGACAGG	Exon	8
NS_S100.For	TCGTTAAGAACGCTGATGACC	Exon	nd
NS_S100.Rev	TCCTCCATAGCTTTAGCAAACAC	Exon	nd
NS_STC.For	GTGGTGTGGCTCGCTCTAAC	Exon	3
NS_STC.Rev	GGGTGGGGTCTCTTCCTCT	Exon	4
NS_VDR.For	CAGAAGAGGAAGGAGGAGGA	Exon	2
NS_VDR.Rev	CTGGTGAGTGGTTGAAGGAG	Exon	3

Initial experiments were conducted with the specific primers to determine optimal cycle number and ensure a good yield of amplicon. Primers complying with requirements were then used to optimise annealing temperature, cycle number and $MgCl_2$ concentrations. The composition of the PCR mix are described in table 5.3, the reactions were performed in a Multigene (Labnet, NJ, US) thermocycler, using the following program: 3 minutes at 95ºC followed by 35 cycles of 30 seconds at 95ºC, 30 seconds at the specified annealing temperature (Table 5.3) and 30 seconds at 72ºC, followed by a final step of 3 minutes at 72ºC. Negative reactions without sample cDNA were also performed to control for contamination. The reaction products were

fractionated on 2% agarose gel stained with ethidium bromide to determine quality and length of amplified fragments.

Table 5.3 – PCR mix conditions and annealing temperature for each gene.

PCR MIX	Target Gene		
	CT I-NaPi STC VDR	Meprin 1A	mtPi OSN OSP S100 11A
REAGENT	CONCENTRATION (VFINAL 25µL)		
$MgCl_2$ (50mM)	1 mM	2,5 mM	1 mM
RB 10x	1x	1x	1x
Fw primer (10pmol/µl)	0.4 mM	0.4 mM	0.4 mM
Rv primer (10pmol/µl)	0.4 mM	0.4 mM	0.4 mM
dNTPs (10mM)	0.08mM	0.08mM	0.08mM
cDNA (1/2)	-		
Taq (5U/µl)	0.025U/µl	0.025U/µl	0.025U/µl
Annealing T (ºC)	56	55	55

5.2.6 Confirmation of fragment identity

In order to confirm that the amplified PCR products corresponded to target genes they were cloned into the vector pGEM-T and sequenced.

Cloning in to pGem-T easy

Cloning of the amplified gene was carried out by incubating 3 µl of the PCR product with 0.2 µl of pGem-T easy vector Promega (VWR, Portugal) using T4 as ligation enzyme (Promega). The reaction mix was incubated at 4°C overnight and used to transfect into competent *E. coli* cells. Transfection was carried out by thawing the cells on ice, adding 1 µl of each ligation reaction gently mixing by pipetting up and down several times and gently stirred with the pipette tip. This mixture was left on ice for 45 minutes followed by 1.5 minutes at 42ºC in a heating block (Grant, Alfagene, Portugal) and incubation on ice. Afterwards transformed *E. coli* were plated on agar LB broth plates (Annex 1) containing IPTG/X-GAL/Ampicilin, in order to select transformed bacteria containing vector and PCR insert. Plates with bacteria were incubated over night at 37ºC and then placed at 4ºC for 7h in order for the colour reaction to develop. 2 white bacterial colonies were selected for each gene and grown overnight in LB broth containing ampicilin (Annex 1) with constant agitation (250 rpm) at 37ºC.

Plasmid DNA isolation

After amplification the plasmid DNA was extracted from bacteria by centrifuging for 5 minutes at 10,000 rpm and retaining the cell pellet. Cells were resuspended in 200 µl of Glucose-Tris-EDTA (GTE) to maintain osmotic balance and 2 µl of RNAse A (10 mg/ml) (Promega) added and the reaction incubated at room temperature for 10 minutes. To lyse the cells 400 µl of 0.2M NaOH + 1% SDS was gently added and the tube mixed by inversion to avoid shearing genomic DNA and then incubated on ice for 5 minutes. 300 µl AcK (3M, pH 5.2) was added to the mixture and incubated on ice for 45 minutes during this step the large and less supercoiled chromosomal DNA and proteins precipitate. After centrifugation at 10.000 rpm, for 10 minutes the supernatant containing the small bacterial DNA plasmids is transferred to an eppendorf containing 1ml of cold EtOH 100% at -80oC for 1h to precipitate. The plasmid DNA is recovered by centrifugation at 10,000 rpm for 20 minutes and the pellet washed with 500 µl of cold EtOH 100% followed by centrifugation at 10.000 rpm for 10 minutes, this procedure was repeated twice. The pellet was air dried at room temperature and resuspended in 50 µl of sterile miliQ water.

To confirm the size of the fragment in the miniprep, it was released from the vector using Promega EcoR1 restriction enzyme, which cuts the vector in the regions flanking the insert (see Annex 1). In brief 2 µl of miniprep where incubated with 1 µl of EcoR1 and 2 µl of buffer H (Promega) in 15 µl of milliQ water at 37ºC for 1.5 h. The reaction product was analysed by 2% agarose gel electrophoresis in the presence of ethidium bromide and quality and length of fragment analysed.

Sequencing

Minipreps containing cloned genes were sequenced in the molecular biology facilities of CCmar (Faro, Portugal), and as the fragments were relatively small a single sequencing primer, T7, gave the full sequence of the insert. To confirm identity of cloned genes and accuracy of sequences they were compared against the NCBI database using Blastn (http://blast.ncbi.nlm.nih.gov) (Altschul et al. 1990) and aligned against the sequence used to design the primers (Table 5.1) using Multi-align software (http://bioinfo.genotoul.fr/multalin/multalin.html). Sequencing chromatograms were

also analysed in Bioedit (Hall 1999) to confirm the quality of the sequencing reaction. The consensus sequences obtained were used to design primers for QPCR assays.

5.2.7 Q-PCR Primer Design

Primers for real-time PCR were selected from within the sequence of the previously cloned gene fragment to be used as a standard for the Q-PCR using Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA), after verifying with Primer Premier software (Premier Biosoft International, CA, USA) that false priming did not occur. The following criteria were used for primer design, amplified product of 50-150 bases in length, primers spanning exon-exon junctions, absence of secondary structure in the template, primers longer than 17 bases, avoid runs of G's and C's longer than three bases, Tm of primers higher than Tm of predicted template secondary structures; no complementarities between the forward and reverse PCR primers, absence of false priming sites within the gene. The overall specificity of designed primers (and probes) was then established by carrying out a BLAST search (Sigma-Aldrich 2008).

5.2.8 Real-Time PCR pre-run tests

Before QPCR is done, a number of optimizations procedures must be performed. The first one is to determine the temperature of primer annealing, Ta. QPCR primers were tested at 3 different temperatures using a test cDNA diluted 1/10. The temperatures tested were chosen based on the predictions of Ta (above it) and Tm (below it) by Beacon Designer and Primer Premier. Negative controls, where water was added instead of cDNA, were also tested at this stage in order to detect contaminations of the template/primers. This control had to be negative before proceeding to the next optimization step.

Since 10 genes were analysed in the study, tests of optimal annealing temperature were performed in 2 separate runs and using a gradient program in the iCycler. In Figure 5.1 the temperature range utilised with primers is represented.

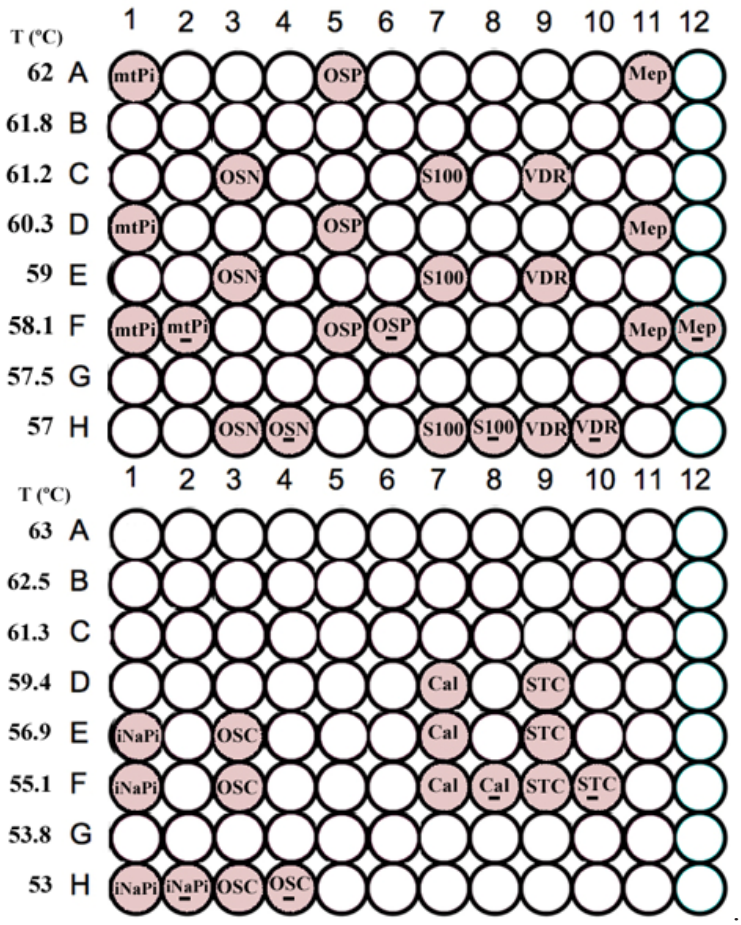

Figure 5.1 – Plate distribution of primers tested and the range of temperatures utilised in the primer optimization study.

For each gene 2µl of of cDNA (1/10) was added to 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Porto, Portugal), and the final concentration of each primer was 100 nM in 20 µL total volume. The PCR reaction took place in a sealed 96 multiwell plate (Biorad, Amadora, Portugal) in a thermocycler iCycler (Biorad). The reaction was initiated with activation of Taq DNA polymerase by heating at 95ºC for 10 minutes, followed by 50 cycles of denaturing (30 sec at 95ºC), primer annealing (30 sec at the test temperatures) (Table 5.4) and extension (30 s at 72ºC). The fluorescence of incorporated SYBR Green was measured in each cycle after the extension step by the iCycler iQ Real-Time Optical Detection System (Bio-Rad, Amadora, Portugal).

On completion of the thermocycle the specificity of the PCR amplification was verified in order to ensure that only specific amplified products were measured. This was carried out using the melting curve method in which the PCR reaction was heated, starting at 65ºC and with a rate increase of 0.5ºC per 10 sec, up to 95ºC and simultaneously measuring fluorescence, allowing detection of nonspecific products. The fluorescence

should decrease gradually with increasing temperature because of increased thermal motion which permits more internal rotation of the bound dye. However, when the temperature is reached at which the double stranded DNA separates the dye dissociates and the fluorescence drops abruptly, this is the melting temperature, Tm, and is determined as the maximum of the negative first derivative of the melting curve. Since primer–dimer products typically are shorter than the targeted product, they melt at a lower temperature and their presence is easily recognized by melting curve analysis (Mikael Kubista et al. 2006). The optimal annealing temperature was selected as that giving the highest amplification at a lower cycle number and a specific melting curve, Figure 5.2 gives an example of the results obtained at this stage.

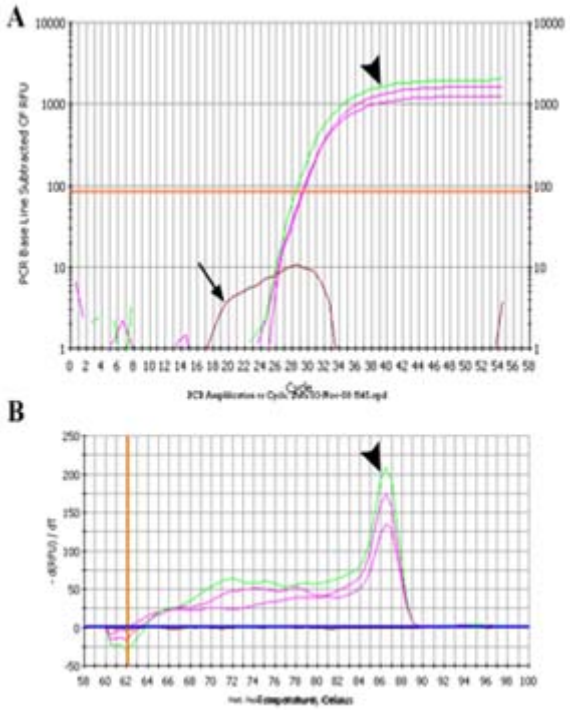

Figure 5.2 – Panel A shows 3 logarithmic amplification curves of the same gene but at different annealing temperatures (green = 57ºC, pink = 59ºC), the arrow (→) indicates the negative control, which has no amplification, and the arrowhead (►) the curve at which the amplification is most efficient. Panel B shows the melt curve for the samples in A, here it is evident that the fluorescence increases at the same temperature correspondent to the curve in A, although this is not always the case.

The identity of the products was confirmed by fractionating them on 2 % agarose gel stained with ethidium bromide to evaluate size and sequencing in the molecular biology facilities of CCmar (Faro, Portugal)

This test also gives an idea of the relative quantity of the transcript present in the cDNA, in principle an amplification at lower cycle number implies a higher quantity, which

could indicate the cDNA dilution and the range of standards to be used in the experiment.

5.2.8 *Preparation of Standard curve*

There are several methods of quantification that can be applied to Q-PCR, the most used is the absolute method, which determines the number of mRNA copies in the sample from a calibration curve; and relative quantification which compares copies of the target mRNA with those of a reference gene, usually metabolic, structural, and ribosomal RNA genes (Sindelka et al. 2006). In the present work standard curve was generated which produced a linear relationship between C_t (threshold cycle) and initial amounts of cDNA, and using their C_t values allowed the initial concentration of mRNA in the experimental samples to be determined (Figure 5.3).

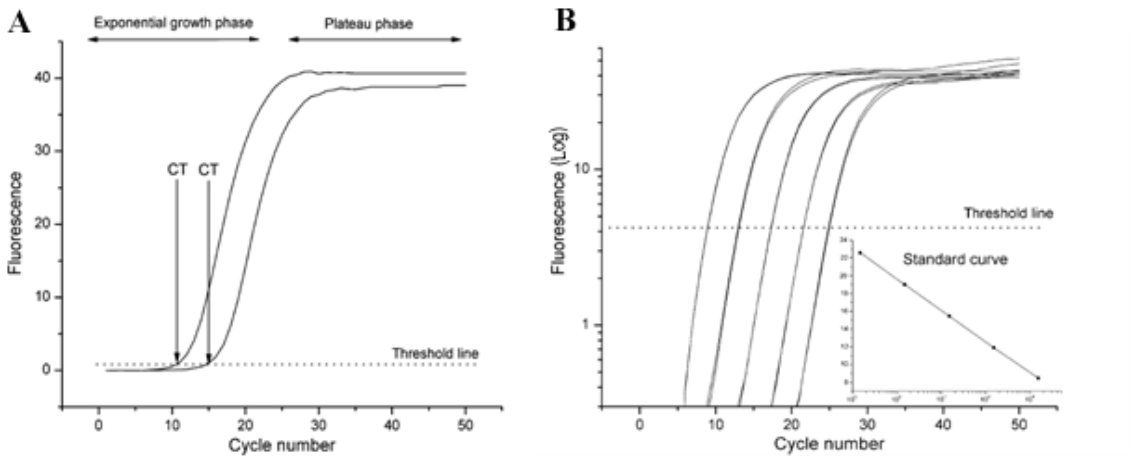

Figure 5.3 - A - Real-time PCR response curves, the threshold level is set sufficiently above background and the number of cycles required to reach threshold, CT, are registered. B - Real-time PCR standard curve shown on a logarithmic scale for five standard samples. The crossing point with threshold line is the CT value for each sample. Inset - the CT values are plotted vs. the logarithm of the known initial number of template copies in the standard samples (Adapted from (Mikael Kubista et al. 2006)).

The standard used was a fragment of double-stranded DNA which contained the target sequence, of known concentration, cloned in *pGEM-T*. This method assumed all standards and samples had approximately equal amplification efficiencies (Wong and Medrano 2005).

Preparation of standards

In order to eliminate secondary structures and ensure miniprep behave in a similar way to the cDNA the purified cloned DNA were linearized using a restriction enzyme which did not cut the insert and was identified with Bioedit (Hall 1999). Minipreps (6 µl) were linearised using 1.5 µl Sal I (Promega, VWR, Lisboa, Portugal) and 10x Buffer D (4 µl, Promega) to a final volume of 40 µl. The efficiency of the linearization reaction was monitored on a 2% agarose gel stained with ethidium bromide.

The linearized product was purified using illustra GFX™ PCR DNA Purification Kit (GE, Carnaxide, Portugal) and resuspended in 30 µl of milliQ water. The purified miniprep was then quantified using Quant-iT™ dsDNA BR Assay Kit (Invitrogen, Barcelona, Spain). In brief, the assay has a fluorophore (in this case PicoGreen) that becomes fluorescent upon binding to DNA, purified recombinant plasmid (1ul) was diluted (1:200) in working solution, vortexed 2-3 seconds and incubated at room temperature for 2 minutes. The fluorescence was read at 480 nm in a Qubit ™Quantitation Platform (Invitrogen) which gave the concentration of dsDNA present in the mixture. To determine the quality of the cloned DNA electrophoresis on 2% agarose gels stained with ethidium bromide was performed. Minipreps were diluted to give a stock containing 0.5 ng DNA/µl and a standard series of, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10ag, 1 ag prepared.

Preparation of cDNA dilutions

In order to guarantee that all the samples fit within the standard calibration curve (Figure 5.5), a curve using serial dilutions of the cDNA was also made. This permitted assay efficiency, precision, sensitivity, and working range to be determined. The dilution series was designed to extend past both the highest and lowest levels of target expected in experimental samples. 5 serial dilutions of cDNA, from 3 individuals of the C group, were made in milliQ filtered sterile water starting at 1:5, 1:25, 1:125, 1:625, 1:3125, the –RT control was also diluted 1:5. For each gene a QPCR run was prepared (Figure 5.4) which contained 5/6 standards, 4/5 cDNA dilutions, -RT control and negative control. The QPCR conditions were the same as indicated for Ta optimization, and used the optimized annealing temperature for primers (Table 5.4).

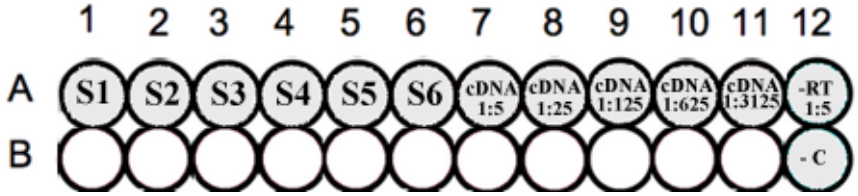

Figure 5.4 – Example for the distribution of the standard curve, cDNA curve and controls in a Q-PCR multiwell plate.

The number of standards and cDNA dilutions varied according to the results of the pre-run for Ta optimization. For highly expressed genes there was no need to use the more dilute standards and for low abundance gene there was no need to use the concentrated standards. The test run formed the basis for a precise selection of standards to be used for the "real quantification" of transcript number in experimental samples.

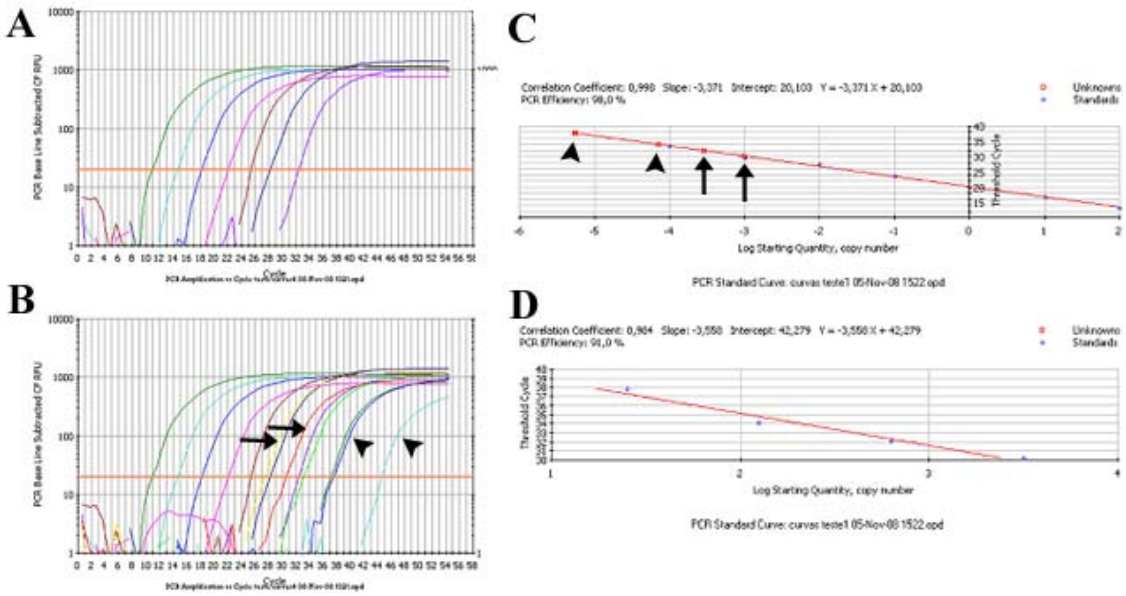

Figure 5.5 – Examples os f the results obtained during the standard curve optimization. A – Standard curve using 7 standards. B – Amplification curve of the standards and the cDNA dilution series. The curves labeled with arrows (→) are inside the standard curves whilst the ones labeled with an arrowhead (►) are outside the standard curve. C – Linear correlation obtained with the standard curve, giving the correlation coefficient, which is a measure of how well the data fits the model and the PCR efficiency. If the PCR is 100% efficient, the amount of PCR product will double with each cycle (80-110% efficiency is generally acceptable). If the cDNA dilutions are plotted (red dots) they are visible over the line and it is possible to determine their concentration when they are within the range of the assay, i.e. between the standards (blue dots). D –Efficiency of amplification of the cDNA dilution series. This will test the specificity and range of the assay but also pipetting accuracy.

5.2.9 Real-Time PCR

After optimization of the QPCR conditions and standard curve these were applied to sample analysis.

Table 5.4 – QPCR primer name, sequence, annealing temperature (Ta), cDNA dilution used and size of the reaction product.

Gene - Primer name	Primer	Ta	cDNA dilution used	Size bp
NS_Calcitonina FW	CCCTCCTCATTGCCTATTTCC	59	1/1	184
NS_Calcitonina FW	GCTTGTTGCTCCAGTTCCTC			
NS_INaPi_FW	TGGGTGGAAGGCAACAAGAC	59	1/10	101
NS_INaPi_RV	AGGTCAGGCAGGTTAGCATAGG			
NS_Meprin1A_FW	GAGACCTACAGACAGGGCAACAG	60	1/10	146
NS_Meprin1A_RV	GTCCCACCAGATGTCCACATAGTC			
NS_MtPi_FW	CCTGTACCTGGCTGCGTCTG	58	1/10	119
NS_MtPi_RV	ACTGTCTGAGGTTGTTGGCGTAG			
NS_OSN_FW	GCAGGTAGAGACTGGAGACTTCG	61	1/10	121
NS_OSN_RV	TTCCACCTCACACACCTTTCC			
NS_OSP_FW	ACAGCAGCAACAGTAGTGAGGAAG	58	1/10	142
NS_OSP_RV	GGAGCATCGGTAGTAATGGTAGCC			
NS_S100_FW	GGTAGTAACAGAACAAGACATGGAGAC	59	1/10	119
NS_S100_RV	ACGAGAACAACGACGGAGAGC			
NS_STC.For (same as semiQ)	GTGGTGTGGCTCGCTCTAAC	57	1/10	84
NS_STC_RV	GAGTGCTGTAGTACCTGTTGGG			
NS_VDR.For (same as semiQ)	CAGAAGAGGAAGGAGGAGGA	59	1/10	241
NS_VDR.Rev (same as semiQ)	CTGGTGAGTGGTTGAAGGAG			
NS_Bactina_q_FW	ATCTGGCATCACACCTTCTACAA	60	1/10	123
NS_ Bactina_q_RV	CTCAAACATAATCTGGGTCATCTTC			
Tetra18SRT_F1	CGATCAGATACCGTCGTAGTTC	60	1/10	180
Tetra18SRT_R1	CCCTTCCGTCAATTCCTTTA			

Q-PCR reactions to measure target transcripts contained 2µl of the appropriate dilution of cDNA for each gene (Table 5.4), 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Porto, Portugal) and appropriate primers at a final concentration of 100 nM in 20 µL final volume. The PCR reaction took place in a sealed 96 multiwell plate (Biorad, Amadora, Portugal) in a thermocycler iCycler (Biorad, Amadora, Portugal). The reaction was initiated with activation of Taq DNA polymerase by heating

at 95ºC for 10 minutes, followed by 50 cycles of denaturing for 30 sec at 95ºC, annealing for 30 sec at the optimum temperature for each gene (Table 5.4) and elongation for 30 sec at 72ºC. The fluorescence was measured in each cycle after the extension step using the iCycler iQ Real-Time Optical Detection System (Bio-Rad, Amadora, Portugal). After the thermocycling reaction, a melting curve of the products was made by heating, starting at 65ºC and with a rate rise of 0.5ºC per 10 sec, up to 95ºC, with continuous measurement of fluorescence, allowing detection of possible nonspecific products. The assay included a negative control, in which no cDNA was added. All reactions were performed in duplicate to reduce technical variability.

Data Processing

Data from Q-PCR was acquired using iCycler iQ Optical System Software Version3.1 (Bio-Rad, Amadora, Portugal). This software allowed the input of known concentrations of the standards and after manual calibration of threshold position above background fluorescence, it automatically calculated the efficiency, the correlation coefficient and the starting quantity of the gene in study

5.2.10 Normalization

Normalization of gene expression data in absolute quantification was used to correct sample-to-sample variation that may arise due to the amount of RNA extracted from tissue or cDNA synthesis efficiency. The ideal control gene should be expressed in an constant manner regardless of experimental conditions, type of tissue or cell, developmental stage, or sample treatment (Schmittgen and Zakrajsek 2000). However, no universal control gene exists and it is necessary therefore to always validate the expression stability of a control gene for the specific requirements of an experiment prior to its use for normalization (Bustin 2000; Gibbs et al. 2003). In the present experiment 2 genes where evaluated for utilization as reference genes, 18S and β-Actin.

5.2.11 Data analysis and Statistics

The starting quantities of the analysed genes in each individual were divided by the respective starting quantity of the normalization gene. The obtained relative value of treatment groups was log transformed in order to normalize the data, and analysed by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

GraphPadPrism v5 (GraphPad Inc., San Francisco, USA) was used for statistics and to plot graphs.

5.3 Results

5.3.1 Cloning of the genes of interest

The candidate genes were successfully cloned and their identities confirmed by sequencing. RT-QPCR was established for analysis of the candidate genes (Table 5.5)

Table 5. 5 – Summary of the candidate P and Ca responsive genes analysed by RT-QPCR. Gene name and accession number (AN), QPCR Ct (Ct), correlation factor (R) and efficiency (%E).

Name	AN	Ct	R	E%
Calcitonin 1A	AB094135.1	31.6	0.997	86.5
INaPiIIb	AF297184.1	27.4	0.994	93.8
Meprin1A	BX084554.3	24	0.995	93
MtPi	CA345770	25.8	0.998	94
Osteonectin	U25721.1	21.7	0.997	92.8
Osteopontin	AF204760.1	26.8	0.998	84.1
S100 11A	EE605180	24	0.996	85.7
STC	AF326318.1	28.7	0.997	95.7
VDR	AY526906.1	31	0.999	82.7
β-actin	AB196465.1	20	0.998	76.3
18S	AF243428.2	13	0.997	88

5.3.2 Expression profile of the normalization genes

In order to select the most appropriate gene for normalization, two of the most commonly used reference genes, 18S and β- actin were analysed.

The results showed that the mRNA expression of both genes varied considerably between D11 and D28 (Figure 5.6). Nevertheless their expression was constant within the same age group, allowing comparison of gene expression in trout fry subject to the experimental diets. In order to assess which of the reference genes was the most reliable, the genes were tested for expression variability among the experimental groups. The normalization gene was selected by analysing expression variability using

the Normfinder function on Genex (www.multid.se/genex) and β-actin was chosen as the best normalization gene (Table 5.6).

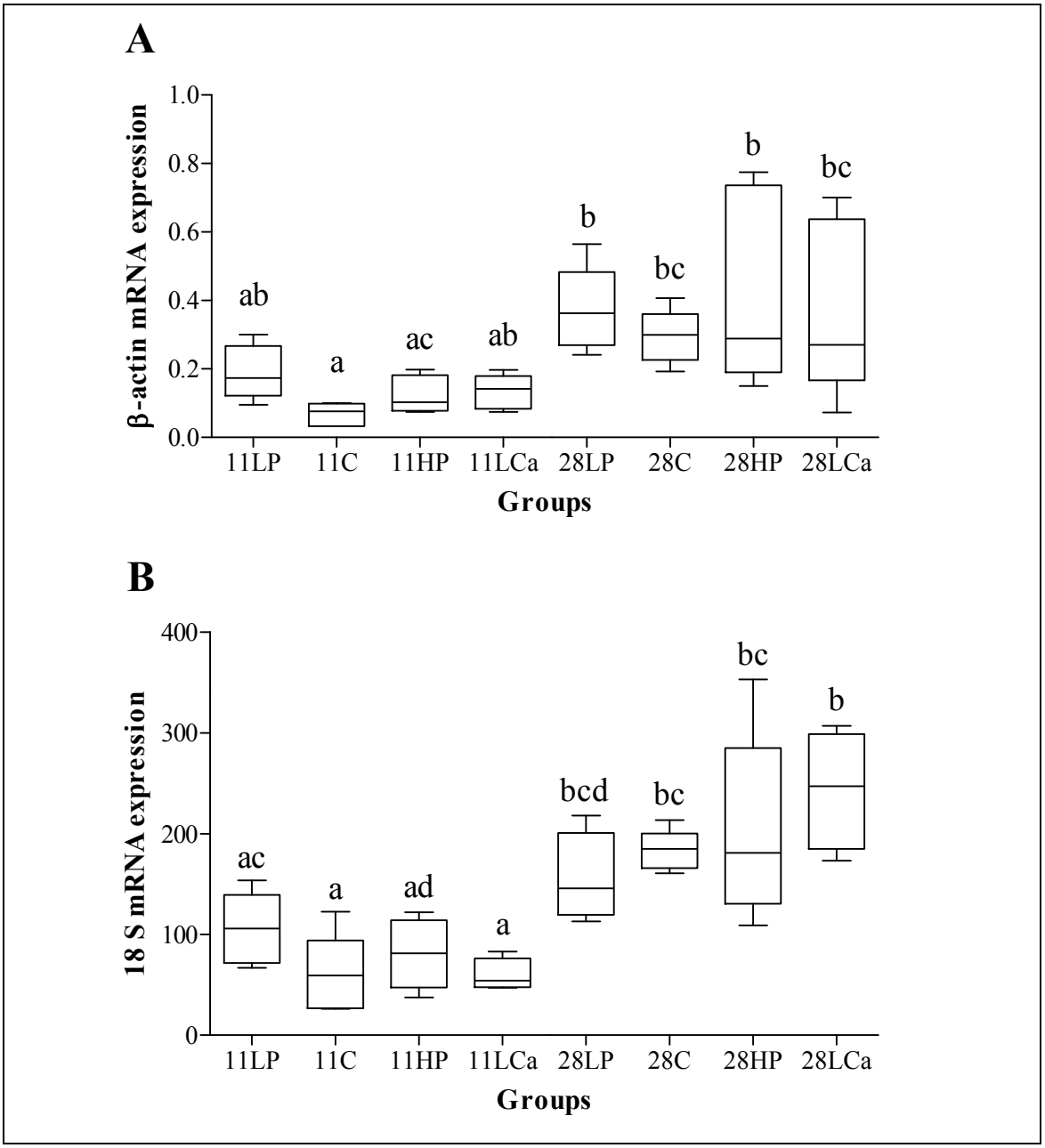

Figure 5.6 – A - Relative expression of *β*- actin mRNA. B - Relative expression of 18S mRNA. Statistical analysis included all experimental groups at D11 and D28 and was performed with 1 way ANOVA (Tukey's multiple comparison test). Different letters reflect significant statistical differences.

Table 5. 6 – Normfinder results, analyzing the variability of potential normalization genes, 18S, *β*- actin and MtPi as an outlier gene.

Gene Name	**Variability**		
18S	0,0453	**Best combination of two genes**	18S and *β*- actin
β- actin	0,0232	**Variability for best combination of two genes**	0,0255
MtPi	0,0279	**Best Gene**	*β*- actin

When the group variability is plotted (Figure 5.7), it is clearly evident that within groups *β*- actin has the best performance as a normalization gene as it has least variability.

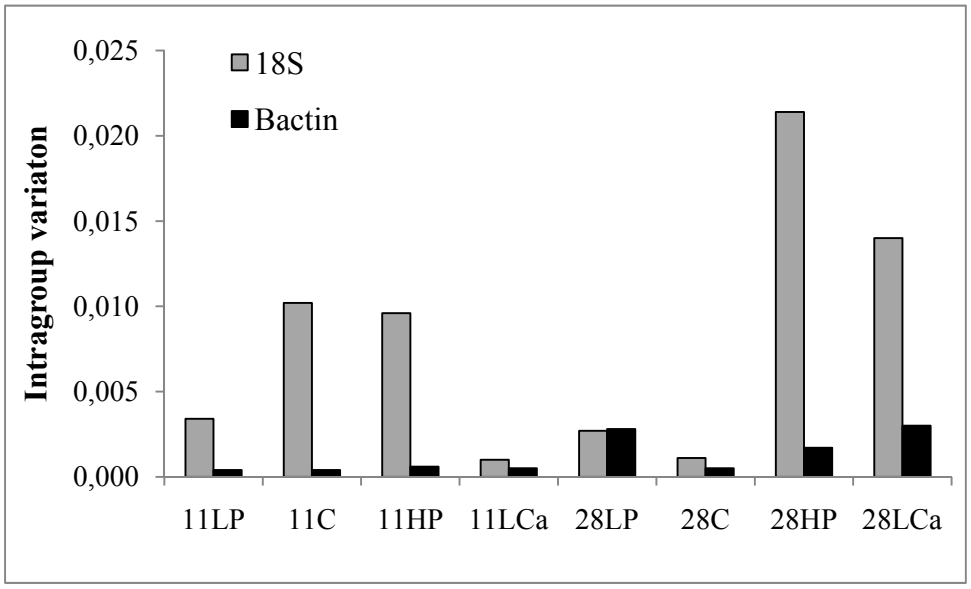

Figure 5.7 - Intragroup variation of 18S and *β*- actin obtained using the Normfinder function on Genex software.

In view of these results and since comparison between different age groups was not feasible using the normalization genes selected, the expression analysis of the genes of interest was made only by comparing groups from the same time point. It will be important in the future to identify a normalization gene appropriate for comparison of the different age groups analyzed.

5.3.3 Candidate genes

Calcitonin 1A

The transcript abundance of calcitonin-1A at D11 was not significantly modified by experimental diet (Figure 5.8), but at D28 both HP diet ($p < 0,05$) and LCa diet ($p < 0,01$) showed a significantly lower mRNA expression than the C group.

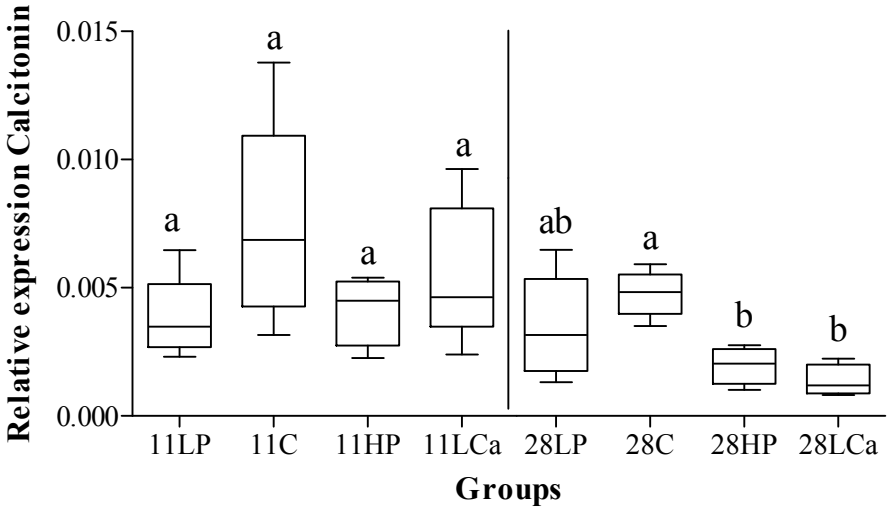

Figure 5. 8 – Relative expression of calcitonin mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

Stanniocalcin 1A

In this experiment STC-1A transcript abundance proved to be very responsive to Pi levels with, HP and LP showing opposite responses at D28.

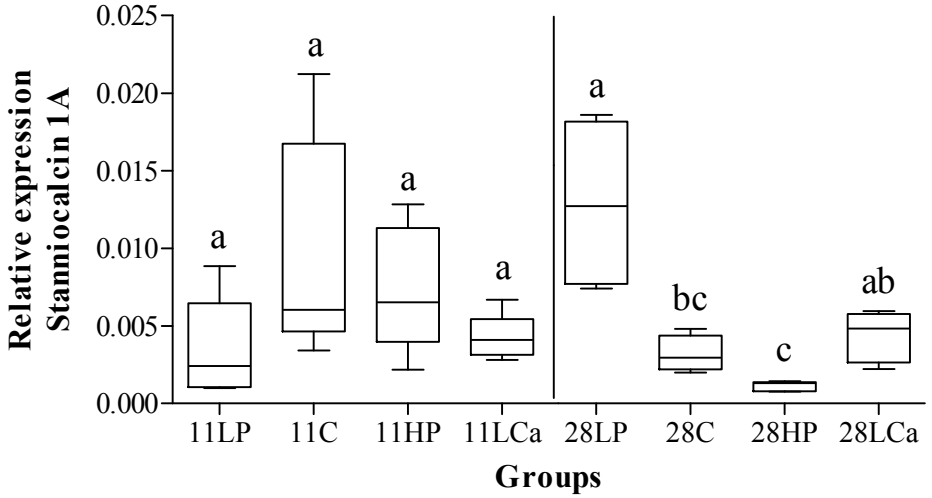

Figure 5.9 – Relative expression of stanniocalcin 1A mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

STC-1A mRNA expression was higher in LP group compared with C ($p < 0,05$) and HP groups ($p < 0,0001$), and there was also a notable difference between HP and LCa groups ($p < 0,05$) (Figure 5.9) At D11 no modification of the expression of stanniocalcin 1A was caused by different mineral contents of the diet.

Meprin 1α

Meprin 1α had a high abundance relative to other gene transcripts measured in the study. No differences were detected in the mRNA expression of meprin 1α, although by D28 the C group has such a large variability that this probably explains the absence of difference although its significance remains to be established.

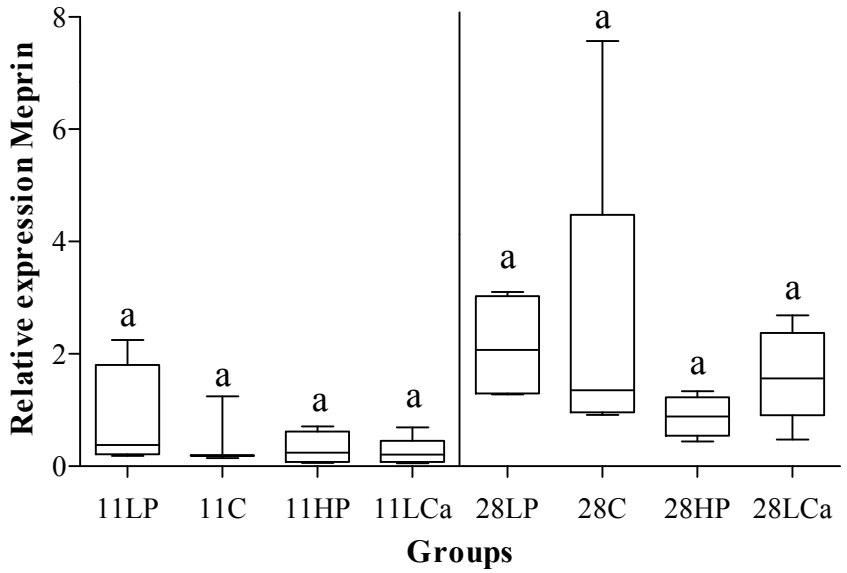

Figure 5.10 – Relative expression of meprin 1α mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups.

INaPiIIb

INaPiIIb has been shown to be modified in restricted P fed adult trout, and it has been considered as a potential marker gene for P deficiency. Interestingly in this experiment at D11 the LCa group appeared to have the lowest abundance of INaPiIIb transcripts and its levels were significantly different from C ($p<0,0001$) and LP ($p<0,0001$) groups (Figure 5.11). HP group had a reduction in of the NaPiIIb transcripts but it was not significantly different from any of the other groups.

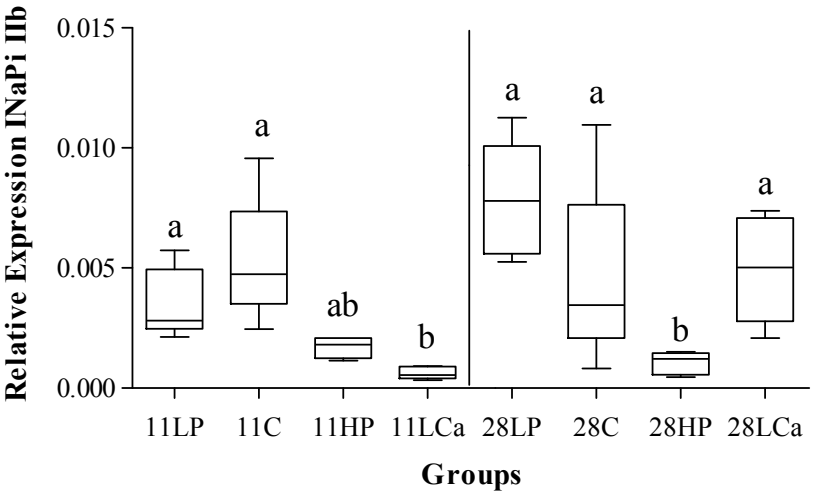

Figure 5.11 – Relative expression of INaPi IIb mRNA at 11dpsf and 28 dpsf. N=5. Normalization was performed using β-actin as the reference gene. 1 way ANOVA (Tukey's

multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

At D28 INaPiIIb transcript abundance was most modified in the HP group which had decreased expression relative to LP ($p<0.0001$), C ($p<0.05$) and HP ($p<0.01$). The LCa group had smilar transcript levels of INAPi IIb mRNA to LP and C at D28.

Vitamin D receptor

The transcript abundance of the Vitamin D receptor was similar in all trout at D11 (Figure 5.12).

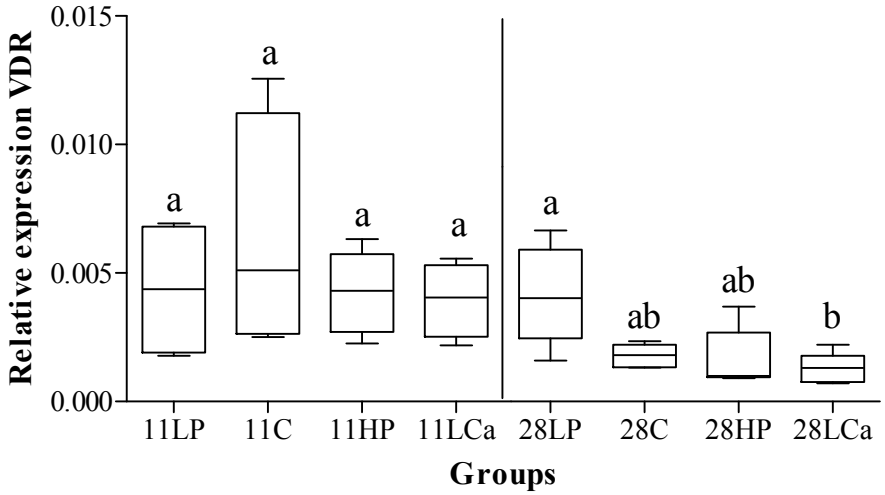

Figure 5.12 – Relative expression of VDR mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups with the same letter did not differ significantly and those with different letters were significantly different cannot be compared as the reference gene was different between the two age groups.

Vitamin D receptor transcript abundance was much more variable between experimental groups at D28. The LCa group had a significantly lower expression of the Vitamin D receptor than the LP group ($p < 0.05$). In the LP group the transcript abundance of Vitamin D receptor tended to be higher than control although not statistically significant.

MtPi

The renal mitochondrial phosphorus carrier was an abundant transcript and its expression was not affected by P or Ca availability in the diet (Figure 5.13). This gene

transcript had an almost constant expression level between all experimental groups and this was true at D11 and D28.

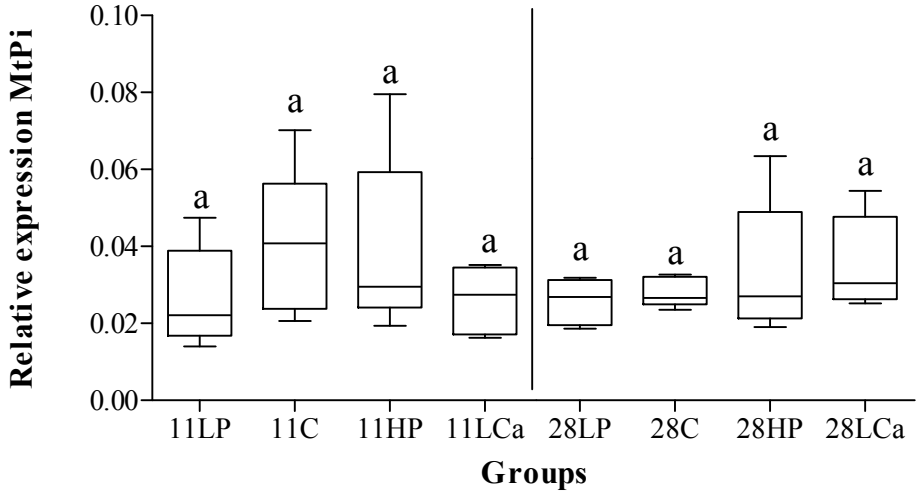

Figure 5.13 – Relative expression of MtPi mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups.

Osteopontin

The osteopontin transcript had a fairly abundant expression and it was highly variable in individuals in the same group transcript in both D11 and D28 trout fry (Figure 5.14). At D11 the C had a remarkably low variation relative to the other groups, suggesting the treatments had an effect but it was not consistent and hence no significant increase or decrease was observed. In the D28 group both the C and HP group had a lower variability relative to the LP and LCa group (Figure 5.14). The high variability in Osteopontin transcript abundance may be a reflection of the developmental variability observed in the analysis of skeletal ontogeny (chapter 3).

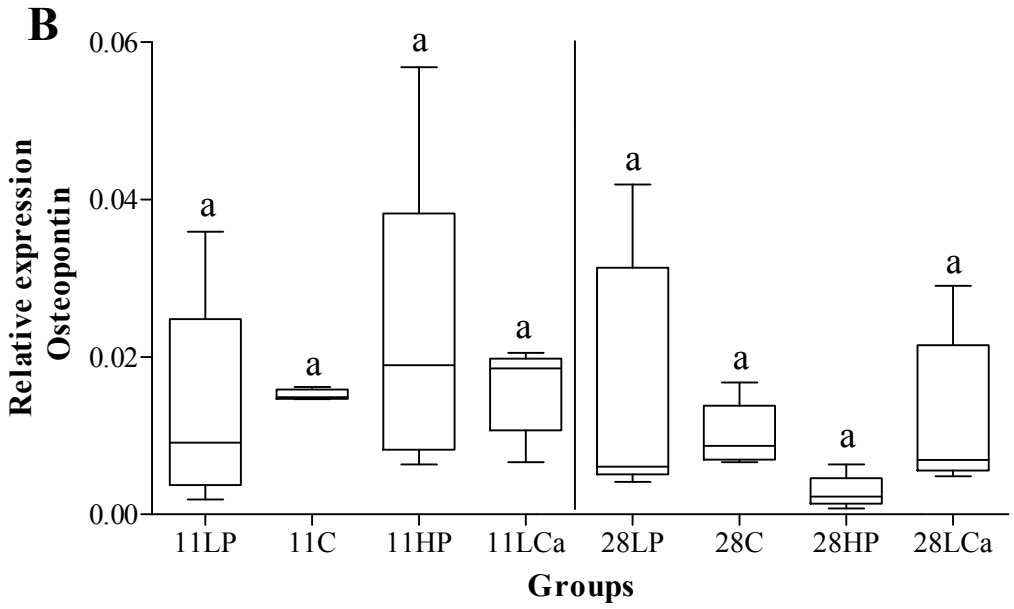

Figure 5.14 – Relative expression of B – osteopontin mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). Different letters reflect significant statistical differences. The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

Osteonectin

Of the gene transcripts linked to skeletal formation only osteonectin presented significant differences between groups. At D11 when the skeleton was at a very early stage of development osteonectin transcript abundance was similar in all group, at D28 the HP group had a significantly lower expression than LP ($p<0.05$) and C ($p<0.05$) (Figure 5.15).

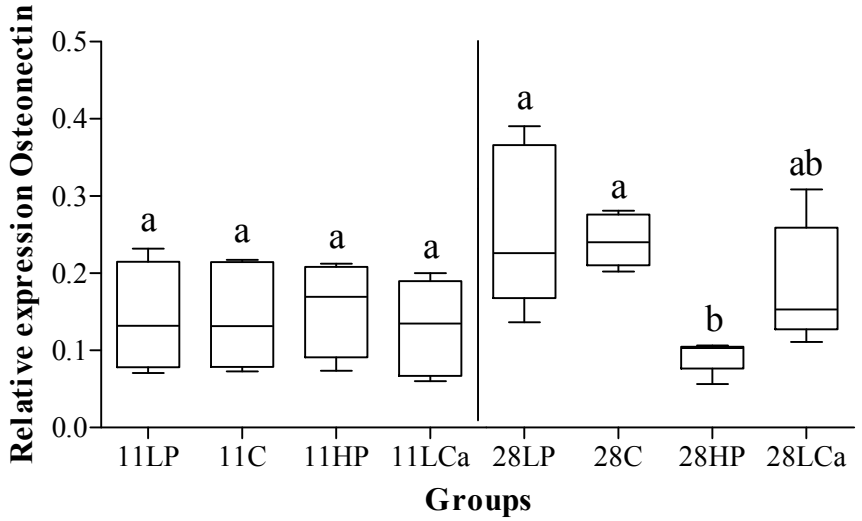

Figure 5.15 – Relative expression of osteonectin mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

S100 11A

At D11 the LP group shows a significantly lower level of S100 11A mRNA relatively to the control group ($p < 001$) showing that the expression of this gene is affected by low dietary phosphorus, but by D28 no difference was detected.

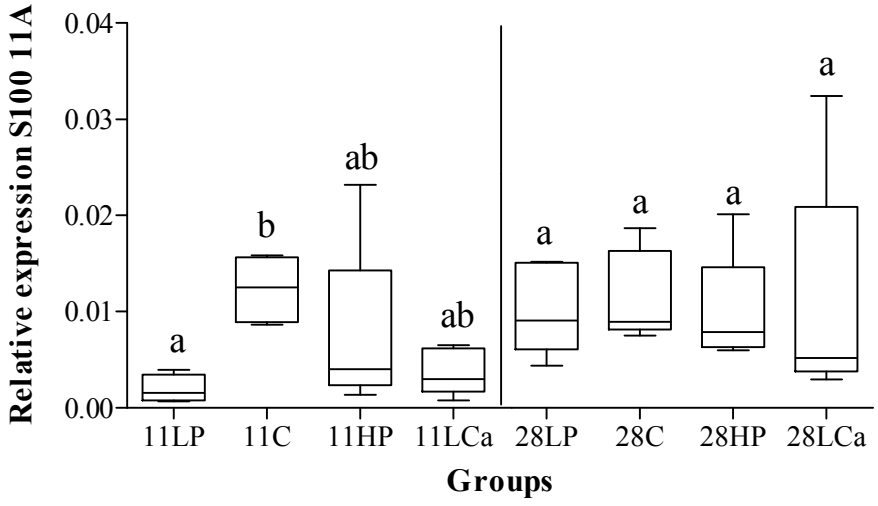

Figure 5.16 – Relative expression of S100 11A mRNA. N=5. Normalization was performed using β-actin as reference gene. 1 way ANOVA (Tukey's multiple comparison test). The two age groups cannot be compared as the reference gene was different between the two age groups. Groups with the same letter did not differ significantly and those with different letters were significantly different.

5.4 Discussion

The data presented here shows the effect of dietary phosphorus (P) and calcium (Ca) on gene expression in trout fingerlings. The objective was to determine if the genes previously reported to be modified by changes in available dietary phosphorus in adult trout are also responsive in trout fingerlings. The effect of modification of available phosphorous and calcium at a stage of development when the skeleton is actively forming was also assessed by analyzing the expression of genes encoding skeletal matrix proteins and hormones known to be implicated in mineral homeostasis.

A difficulty encountered in the present study was the selection of an appropriate reference gene (18s and β-actin). The variation in expression of the reference genes selected in the present study for studying gene expression in different developmental stages has previously been described in *Xenopus* (Sindelka et al. 2006) and there are a number of publications that focus only on the methodology for selection of the reference gene during development or under certain experimental conditions (Vandesompele et al. 2002; Radoni and Siegert 2004). In order to maximize the information gathered from the data, the ideal situation would be to test more genes in order to identify one with constant expression irrespective of age, but due to time restrictions this was not possible in the present study. The variability noted in the reference gene expression was also a feature of the target genes and this high intragroup variation has also been noted by previous authors working with rainbow trout who have attributed it to the remarkable genetic variability of the species (Felip 2004; Kirchner et al. 2007). This variability most certainly implies individual metabolic responses which could lead to difficulties when trying to identify differentially expressed genes under experimental conditions. In addition to genetics the individual feed intake may also contribute to the intragroup variability observed in this study which would influence the amount of P and Ca consumed by the fish.

Transcripts encoding the endocrine factors calcitonin and stanniocalcin which play a key role in Ca and P balance and homeostasis were modified. It appears that the diet low in phosphorous (LP) does not seem to trigger an immediate response in calcitonin 1A expression, whereas there seems to be a clear response to a chronic state of both phosphorus excess and calcium deficiency at D28 even taking into account variations

associated with developmental ontogeny of the ultimobranchial gland, (Chapter 4). Calcitonin is described as a hypocalcemic hormone in mammals and also affects P metabolism, but its role in Ca and P homeostasis in fish is not completely clear. Previous reports state that calcitonin can act as an inhibitor of calcium transport in fish gills (Wagner et al. 1997) and that it also plays a hypocalcemic role in calcium absorption via the digestive tract in eels and goldfish (Sasayama et al. 1996). Caution must be taken when interpreting transcript abundance as they may not be directly correlated to protein levels. Nonetheless, the reduction in calcitonin transcript abundance and presumably protein levels would allow the fish an attenuation of inhibited calcium uptake, which would favor an increase in the transport of calcium in the gills and GI tract in trout on LCa diets which provide significantly less calcium than the fish require. Furthermore, the results also imply that despite the large concentration of Ca present in the water in which the fish live, Ca in the diet is important and this has previously been reported in sea bream (Abbink et al. 2006). The HP fish also need to increase calcium uptake from the environment and diet in order to maintain Ca/P balance in the body and this may explain the reduction in calcitonin transcript abundance in this group of rainbow trout fry.

The primary function of STC in fish is to prevent hypercalcemia (increase in serum calcium levels) and high plasma calcium stimulates its secretion. STC lowers calcium uptake by the gills and intestine and reduces calcium movement from the aquatic environment into the circulatory system, thereby reducing serum calcium levels (Lu et al. 1994). A second function of STC in fish is the stimulation of phosphate reabsorption by the renal proximal tubules through an adenosine 38,58-cyclic monophosphate (cAMP)-dependent pathway (Lu et al. 1994), leading to increased levels of serum phosphate. The increased phosphate combines with circulating calcium, thus promoting its deposition into bones and scales and reducing serum calcium levels. This combined effect of STC on calcium and phosphate movement is synergistic in lowering serum calcium levels. In this experiment the high levels of STC-1A transcripts in LP fish, would allow them to decrease Ca transport in order to maintain Ca/P ratio, increase body levels of P by increasing its uptake in the intestine and reabsorption in the kidney and favor bone development. In contrast, low levels of STC-1A transcripts observed in HP fish would increase Ca transport and decrease P absorption in intestine and kidney,

reducing the high levels of plasma minerals caused by the P rich diet, or at least balancing them.

INaPiIIb is increased in P restricted adult trout, and has been proposed as a marker gene for P deficiency (Sugiura and Ferraris 2004a; Kirchner et al. 2007; Sugiura et al. 2007). In the current experiment on trout fry the LP group always had a similar level of expression of INaPiIIb to the control group, which may indicate that P restriction at this age does not influence the transcription of this gene in trout fry. Previous work done in developing rat reports that $INaP_iIIb$ gene expression varied depending on age (Xu et al. 2002). LCa group showed lower expression levels of INaPiIIb than C or LP at D11 but not at D28, which could indicate that at early stages of development calcium availability, seems to influence P absorption. One explanation for the different behavior of INaPiIIb transcripts at the 2 time points analysed in the present study may be that at D11 Ca uptake from the water has not attained its optimal rate due to physiological restrictions and the osmotic balance is therefore maintained by low P absorption. When Ca uptake increases more P is necessary to maintain homeostasis so P uptake from intestine, via INaPiIIb also increases. The decreased expression of INaPiIIb in HP diet (statistically significant at D28) is in agreement with the fact that excess phosphorus in the diet could trigger a mechanism to decrease P absorption in the intestine and reduced transcript abundance may indicate a reduction in translated protein (Sugiura and Ferraris 2004a). In trout Pi transport in the gut seems to occur predominantly in the pyloric ceca (PC) via paracellular diffusion trough PCNaPiIIb (Sugiura and Ferraris 2004a), but due to methodological difficulties the analysis of this gene was not concluded.

Several studies have shown that Meprin 1α is upregulated in the intestine and pyloric ceaca of adult trout after 5 days on a low P diet (Sugiura and Ferraris 2004a; Kirchner et al. 2007; Sugiura et al. 2007) and the effect was maintained up to 20 days when the experiment stopped and indicates this may be a suitable gene marker for chronic dietary P restriction (Sugiura and Ferraris 2004a). In the present experiment however on trout fry no significant difference was noted in Meprin 1α transcript expression between groups at D11 or D28, although the 28C group was highly variable. Meprin belongs to the zinc metaloprotease famiy of astacin, which are thought to be involved in developmental processes. Meprin 1 α is one of the few constituents of this family that is expressed in mature organisms in a tissue specific manner (Bond 1995). Recent

publications indicate meprin subunits are involved in pathological conditions such as ischemic acute renal failure and urinary tract infection (Yura et al. 2009). Due to this it is thought to play a role in the inflammation process that may occur in the intestine of P-deficient fish (Bond 2005; Kirchner et al. 2007). The lack of a significant effect of mineral availability on Meprin 1α transcript abundance in the present study may be related to the fact whole trout fry were used for QRT-PCR analysis so if tissues respond differently to the challenge the overall transcript abundance may not vary. An alternative explanation may be that P restriction during this experiment was not enough to trigger a response, and certainly the efficiency of P uptake was much higher in this group (Fontagne et al 2009, *in press*) and may reflect an overall increase in transcript abundance of other P transporters not studied in the present study. Moreover, it may be that during larvae development meprin has other important functions in development and growth, and therefore the effect of reduced P nutrition was masked.

In fish meprin α expression has been studied in adult zebrafish (Schutte et al. 2007) and it is present in head kidney, epidermis, gills, liver, heart, brain and intestine and the β subunit has a different expression. No studies until now exist about the ontogeny of meprin α expression during fish development. A study in mouse embryos revealed that the expression of meprin α and β is tissue-specific and it is localized in the developing kidney and intestine, as in adult mouse. At earlier developmental stages of mouse meprin is proposed to be important in the formation of the kidney and intestine, possibly related to the control of growth factor concentration or extracellular matrix proteins (Kumar and Bond 2001).

MtPi carrier is also not affected by P availability in the diet, in the present study on trout fry. In fact MtPi had an almost constant expression level between groups and at the developmental stages studied there was no indication that dietary P or Ca had an effect on its expression. This is in contrast to what occurs in adult trout, where it was over expressed in kidney (Sugiura and Ferraris 2004a) in both short-term and long-term P restriction in adult trout. However, a limitation of the present study is that whole trout extracts were used and if tissue had a differential response this would lead to an "apparent" lack of response.

In the present experiment dietary low calcium caused decreased expression of VDR relative to the dietary low phosphorus group, which was slightly increased relative to the control demonstrating that VDR expression has a distinct response to the 2 minerals. It is known that $1\alpha,25(OH)_2D_3$ increases mineral transport in rainbow trout gills, and vitamin D deficiency is associated with decreased growth and mineralization of the fish skeleton. Increase in $1\alpha,25(OH)_2D_3$ and VDR concentrations have been noted in salmon migrating from freshwater to seawater suggesting that synthesis of these compounds undergo alterations depending on ambient calcium concentrations (Lock et al. 2007; Qiu et al. 2007). In mammals subject to a low Ca^{2+} diet the stimulation of VDR gene expression when 1,25(OH)2D3 is injected apparently does not occur, as a result of transcriptional repression by the increased levels of parathyroid hormone (PTH). This suggests that the increase in bone mass observed by $1\alpha,25(OH)_2D_3$ administration depends ultimately on the availability of Ca^{2+} and P in the diet (Ferrari et al. 1998). If this is the case also in fish is less clear but in the present experiment reduced VDR transcript expression occurred in trout fry on LCa diets, whilst a slight increase in expression was seen with LP diets and the effect may be explained by the potential regulation of P reabsorption by Vitamin D_3 in the kidney (Sugiura and Ferraris 2004a). It has been hypothesized that $1\alpha,25(OH)_2D_3$ influences transcellular Pi transport in the intestine by modifying INaPiIIb transcription and/or affecting its activity at a posttranscriptional level (Bringhurst and Leder 2006). In adult trout kidney this gene was modified by P restriction in a similar way to suggesting co-regulation (Sugiura and Ferraris 2004a). However, this response was not clear in the present experiment on trout fry, suggesting other factors at this stage of development may be important. Furthermore results in VDR null mice show that intestinal Na/Pi cotransport adaptation to a low Pi diet occurs independent of vitamin D (Segawa et al. 2004).

S100 11A is a calcium binding protein and its transcription was highly responsive to phosphorus deficiency in the trout kidney (Sugiura and Ferraris 2004a) and it has been proposed as marker for chronic P deficiency. In the present study on trout fry the response of S100 11A was different and P deficiency led to downregulation at D11, which returned to control levels at D28. The fact that the expression is only modified at D11 but not at D28 suggests the response of the gene is transient. This protein is thought to be involved in the calcium dependent regulation of actin filaments with consequent regulation of actomyosin ATPase and interaction with annexin A1 through

calcium-dependent membrane aggregation, important for cell vesiculation and membrane cross linking and cytoskeletal organization (Bianchi 2003; Santamaria-Kisiel et al. 2006; Cecconi et al. 2007). Nuclear translocation of S100A11 causes diminished cell proliferation, suggesting the protein might be involved in the contact inhibition of cell growth (Donato 2003). A more recent proteomic study on chronic hypoxia in the kidney showed that it aggravates renal injury, and increases expression of a series of proteins amongst them is S10011A (Son et al. 2008). Further insight on this protein function in the kidney, is therefore crucial to determine why it seems to be affected by P deficiency.

The genes analysed encode proteins which are involved in bone remodeling, resorption and formation and the variation in their mRNA levels does not seem to reflect the variation in the developmental status of the skeleton described in chapter 3, in which D11 and D28 LP were delayed in relation to the other groups. The two ECM proteins analyzed in this study were largely unaffected by low phosphorus or calcium in the diet. Nevertheless osteonectin was very significantly downregulated at D28 in high phosphorus fed animals. This protein is expressed in kidney tubules in fish, and its expression is downregulated by PTHrP (Estêvão et al. 2005; Redruello et al. 2005a) so the decrease in expression may be a result of the hypercalceamic role of PTHrP (Guerreiro et al. 2007) to increase Ca uptake and balance Ca:P in this group. Future analysis of the PTHrP gene in these specimens would be of great value to further clarify its role in Ca and P homeostasis and also its putative regulatory role in osteonectin expression. It will also be important to determine how gene transcription and protein synthesis/deposition are related, as these proteins accumulate and integrate into a relatively stable matrix and so transcript abundance may bear little relationship with protein accumulation.

5.5 Conclusion

The expression of the hormones CT and STC is affected by dietary mineral availability, only at D28. Stanniocalcin's low expression is in accordance with the previous known functions in reducing calcium transport and increasing phosphate reabsorption. Calcitonin shows decreased expression with both high P and low Ca demonstrating in this experiment an inhibition of its hypocalcemic role. The study of other endocrine factors, such as thyroid hormones, PTHrP and phosphatonins would give a more complete understanding of the involvement of the endocrine system in the adaptation to altered dietary minerals in vertebrates.

Of the candidate gene markers for low P only S10011A was downregulated and only at D11 in the diet LP, while INaPiIIb was downregulated at D11 by low calcium. At D28 both INaPiIIb and OSN were downregulated in high phosphorus diet. Meprin 1a, MtPi, OSP and VDR did not show any response when compared to the compound fed animals.

The lack of significant differences obtained may result from the observed intragroup variation, the fact that the analysis where performed in whole body extracts instead of focusing on the main osmoregulatory tissues, kidney, gills, intestine may have lead to the discrepant results, since many of the candidate genes have other known functions in fish. Nevertheless the data gathered from this experiment indicate that the adaptive response to dietary mineral restriction (eg. P and Ca) as exemplified by modification in gene transcription may differ between trout fry and adults. The study indicates that gene markers found in adult fish cannot be transposed directly to analysis of developing individuals and this is probably related to a complex range of factors such as, the maturational status of tissue, the physiology of larvae, the nutritional requirements etc.

PART 6

GENERAL DISCUSSION

6.General Discussion

Calcium and phosphorus play a critical role in diverse biological processes, and their regulation is essential for organism health. In the present study, the adaptive response to Ca^{2+} and P dietary availability was evaluated taking into account the effect on skeletal ontogeny and skeletal density in trout (*Oncorhynchus mykiss*). The expression of previously known Pi responsive genes, along with calciotropic hormones STC, CT and extracelular matrix proteins (osteonectin and osteopontin) was evaluated by RT-QPCR. Our objective was to determine if the same genes that are affected by dietary phosphorus in adult trout are already modified in fingerlings, and try to relate these findings with skeletal development and features of the skeleton at the juvenile stage.

Lowering dietary P in trout fed originated individuals that were both hypophosphatemic and hypocalceamic, presenting delayed skeletal ontogeny, lower bone mass, and increased incidence of malformations. This group displayed similar dry matter content with lower ash content compared to other groups of trout fry and these are characteristics of hypophosphatemic fish which present an increased lipid level (Yang et al. 2006). Adipocytes originate from the osteoprogenitor cells through the action of PPARγ2 transcription factor (Marie 2008) so there is a possibility that under P restriction the adipocyte differentiation pathway is favored it is a shared hypothesis that adipocyte differentiation occurs at the expense of osteoblast differentiation (Lecka-Czernik and Suva, 2006).

From the previously identified P responsive genes only S10011A showed to have a transient response to P restriction. There is still no recognized active role for this protein in P metabolism and the fact that it is modified may be a consequence of the deficiency status. Nevertheless it has been recently described the presence of high levels of this protein during kidney injury (Son et al. 2008) so it would be very interesting to investigate this further. The fact that the increase in expression is transient, although levels of body P at D28 remain below normal may direct to a role in adaptation. The CS showed increased volume and stanniocalcin was also elevated in these individuals emphasizing the role of STC as a promoter of P absorption in the intestine and resorption in the kidney (Wong et al. 1998; Yoshiko et al. 2003). No alterations in UBG

morphology and activity or calcitonin expression were noted, so although calcitonin action at the level of the gill may be mediated by STC in salted water hypocalceamic fish (Wagner et al. 1997) and although P restricted individuals also have low body Ca^{2+} that relation is not clear in this experiment.

In this work the limitation of calcium and phosphorus in the diet caused a prolonging of the duration of mineralization. In mammals in the transition of cartilage to bone there is a period, when the cartilage ECM is destroyed by osteoclasts to give rise to bone ECM and mineralization (Nakashima and de Crombrugghe 2003) and its initiation is detected by the loss of the characteristic staining with alcian blue. The mechanisms by which this happens in teleosts are still not clear but it seems to bear similarities to mammals in that osteoclasts have been identified in vertebrae centra and arches of developing seabream (Estêvão et al. 2009) and the loss of affinity is observed also on the present experiment. Although the expression of ECM proteins is not affected by restriction of P it may affect its function. The level of phosphorylation of OSP apparently as an impact on its function, when 40% of its phosphorylation sites are filled mineralization does not occur, when 95% of its sites are filled, it appears to promote hydroxyapatite formation (Gericke et al. 2005). So analyzing the phosphorylation levels of OSP would clarify its activity under P restriction. During the mineralization process several molecules are in interplay but the limiting factor seems to ultimately be the availability of Ca and P necessary for the formation of hydroxiapatite.

Evidence suggests that Pi homeostasis is a system with limited redundancy this means that pathophysiological events may need to target only a restricted number of the critical elements of this network to eventually lead to disturbed Pi homeostasis and phenotypic manifestations, such as osteopenia or osteoporosis (Kiela et al. 2009). As we can see in the LP fish in the present study calcium body content was never different from control individuals except for P deficient fish, whilst phosphorus content varied in conformity with the ingested amount, suggesting that even in lower vertebrates the mechanisms that control P are less robust and also less known. The mechanisms of adaptation in P restricted individuals were not sufficient, even at long term (87 days), to compensate the deficiency. This emphasizes the need for further analysis and understanding of this regulatory network.

Other genes of interest to determine the full adaptive response of larvae to the effect of dietary P restriction would be NaPi-IIa responsible for kidney reabsorption of P as even relatively minor changes in its expression and protein activity translate into clinically significant alterations in Pi homeostasis and phosphaturia (Kiela et al. 2009) and PCNaPiIIb which is the main contributor to P dietary absorption (Sugiura and Ferraris 2004), that due to time limitations where not analyzed in the present work.

Calcium restricted fish always maintained mineral levels similar to control fish, and their bone density at the end of the experiment was also similar to control. The adaptive mechanisms worked to a certain extent; nevertheless these individuals did present a decreasing growth rate, contrary to the P restricted group that was hypophosphatemic and hypocalceamic, and also high incidence of malformations, mainly in the vertebral column robustness. They presented smaller vertebrae and possibly as a consequence high incidence of kyphosis. The defective condensations can have important consequences in the outcome of the skeletal structure, a small condensation may result in an abnormally small bone or even absent bone (Hall and Witten 2007). In what way dietary minerals influence mesenchimal condensations, to my knowledge is not documented but it seems from this experiment that Ca^{2+} restriction may affect the morphology of the bone structure, but not necessarily its calcification level. The low expression of calcitonin observed in these individuals may have as effect an increase in osteoclasts activity and favor resorption as a way of releasing Ca^{2+} from the recently formed bone (Huebner et al. 2008) and in that way restore plasmatic levels.

It has been described that consumption of a excess of dietary phosphorus, when calcium intake is adequate or low, causes decrease weight, hypocalcemia, hyperphosphatemia, secondary hyperparathyroidism with enhanced bone resorption and bone loss in several animal models which could lead to bone fragility and malformations (Calvo 1993; Tani et al. 2007). Curiously this did not occur during this experiment, for it may contribute the high mortality and/or the fact that the between D11 and D28 the % rate of P and Ca accumulation in the body of HP animals decreases whilst on the other groups it increases, to this may contribute the decreased expression of so many of the candidate genes in this group INaPiIIb, STC, CT, OSN. The efficiency of P utilization was very low in these individuals throughout the experiment, and after D28 the calcium accumulation increased in order to compensate for the high body P. This is also

confirmed by the low expression of both the anti-hypercalceamic hormone STC (although not significantly) and the hypocalceamic hormone CT in these individuals. The body %P excess observed in this group by D11 may drive to a reduction in mineral intake and accumulation in the bodyin order to decrease the resources used to form bone and avoid overcalcification. Previous papers describe that when excess P was fed to weanling mice it demonstrated systemic toxicity with a decrease on serum Ca and consequently detrimental effects in body weight, and brain and lung development (Jin et al. 2006; Jin et al. 2007). Possibly, the low degree of development of trout when the experiment began, together with very high levels of phosphorus, rendered systemic toxic effects on the larvae. The remaining fish were probably the fittest, so the results obtained for this group are very possibly biased. Nevertheless this group showed normal bone mass and the lowest number of abnormalities incidence.

The increased expression STC mRNA in LP group is in accordance with the results obtained from CS morphometry, where a noticeable increase in CS number was seen. The same did not happen in the remaining groups which showed a decreased CS volume but no concomitant decrease in mRNA expression. This fact may derive of the observed variability both in morphology as in mRNA expression, furthermore the analysis of mRNA and morphometry was not done in the same individual, and this would have been the ideal situation. Nevertheless when mRNA expression is significantly higher than the control situation the effect in morphology is notably visible. The comparison between UBG nuclear activity and calcitonin mRNA expression was not consistent. High nuclear area did not have as outcome in higher mRNA synthesis. Although the patterns of protein expression may be different from that of mRNA abundance in the case of UBG to establish activity based solely on morphology may lead to incorrect analysis.

In the developmental stages in study the expression of these genes may not be under the influence of nutritional P, or that the deficiency from such an early stage triggers other types of adaptive mechanisms that do not involve the same genes as seen in adult trout. Nevertheless bone development and deformities which are associated with nutrient deficiencies and toxicities in fish can provide an excellent model to study gene action, cell differentiation, morphogenesis, deposition of calcium, phosphorus and other trace elements in cartilage and skeletal tissues. (Lall and Lewis-McCrea 2007). Fish systems

could be of enormous benefit to skeletal research because they are tractable, exhibit a diverse range of deformities, are free from an appendicular skeleton, and substantial genomic resources have been developed for several species (Gorman and Breden 2007).

The work developed during this thesis allowed the development of an *in vivo* model for hypophosphatemia which produced an osteopenic if not even osteoporotic organism. In order to improve this study it would be essential to establish a new reference gene in order to ascertain the differential expression over the 2 time points in study. It would also be of great interest to increase the panel of in vivo P responsive genes in order to obtain a more complete view of P deficiency status, ranging from phosphatonins to PTH- like peptides and broaden the range of genes involved in bone development, like osteocalcin, and alkaline phosphatase and some of the transcription factors involved in osteoprogenitor cell differentiation. Analyze cellular components of bone, and ECM composition would also help to elucidate the mechanisms involved in the prolonged transition period observed in the P deficient fish.

In conclusion this study indicates that modifications in the dietary Ca and P during development significantly modify the ontogeny and density of the skeleton. The availability of Ca and P also led to modification in endocrine factors presumably to ensure maintenance of mineral homeostasis. It seems likely that changes in hormone production may be responsible for changes in transcript abundance of specific transporters/binding proteins of Ca and P although in the present study it was not possible to test this hypothesis.

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ANNEX 1

4% PARAFORMALDEHYDE PH 7.4 (1L)

40 g PFA (Sigma, Madrid, Spain)

900 ml of sterile milliQ H_2O

100 µl NaOH

Heat solution to 65ºC until complete dissolution of the paraformaldehyde powder. After it is left to cool and 100 ml of sterile 1M phosphate buffer pH 7.4 is added. The final pH must be checked and corrected it if necessary.

BOUIN-HOLLANDE

Stock solution:

2.5 g copper acetate

4 g picric acid (Merck, VWR, Lisboa, Portugal)

Dissolve in 100 ml distilled water

Just before use add:

100 ml stock solution

10 ml 37% Paraformaldehyde (Merck, VWR, Lisboa, Portugal)

10 ml saturated solution of mercuric chloride (Merck, VWR, Lisboa, Portugal)

PHOSPHATE BUFFER 1M PH 7.4 (PBS) (250 ML)

31.15 g $Na_2HPO_4.2H_2O$ (Sigma, Madrid, Spain)

11.70 g $NaH_2PO_4.2H_2O$ (Merck, VWR, Lisboa, Portugal)

The compounds are dissolved in milliQ H_2O. Adjust the pH to 7.4 and complete the volume to 250 ml.

DEPC WATER (500 ML)

50µl of DEPC (Sigma, Madrid, Spain) are added to 500 ml of milliQ water. The mixture is agitated and left to stand for at least 2 hours before autoclaving.

EDTA 0.5 M PH 8.0 (1L)

186.12 g EDTA.Na_2.2H_2O (UBS, Amersham Life Sciences, Lisboa, Portugal)

to 600 ml of distilled water. The pH is adjusted to 8.0 with NaOH pellets (Merck, VWR, Lisboa, Portugal), after which the final volume is adjusted to 1 liter.

PARAFFIN EMBEDDING

Paraffin embedding was performed in an automatic tissue processor (Leica TP 1020) with the program:

70% Ethanol:	10 minutes
95% Ethanol:	30 minutes
95% Ethanol:	30 minutes
100% Ethanol:	1 hour
100% Ethanol:	1 hour
Ethanol: Xylene (1:1):	1 hour
Xylene:	1 hour
Xylene:	1,5 hours
Xylene:Paraffin (1:1):	2 hours
Paraffin:	2 hours

In the end of the program, paraffin blocks were prepared with low temperature melting paraffin (Merck, VWR, Lisboa, Portugal).

APES TREATMENT OF SLIDES

APES, aminopropyltriethoxysilane (Sigma, Madrid, Spain). Coating with APES turns slides adhesive in order to facilitate adhesion of fixed, paraffin embedded tissue. Slides are immersed in 1% acid alcohol (% (v/v) concentrated HCl, 70% Ethanol, 29% H_2O) for 30 mins. The slides are rinsed in tap water, followed by distilled water and allowed to dry. The slides are then immersed in acetone (Merck, VWR, Lisboa, Portugal) for 10 minutes followed by 2%(v/v) APES in acetone for 5 minutes and a rinse of distilled H_2O.

PBS (T) (1L)

0,2g KCl (Sigma, Madrid, Spain)

8,0g NaCl (Sigma, Madrid, Spain)

0,2g KH_2PO_4 (Sigma, Madrid, Spain)

1,15g Na_2HPO_4 (Merck, VWR, Lisboa, Portugal)

1ml Triton X-100 (Sigma, Madrid, Spain)

The reagents are added to 900ml of H_2Od and agitated until they are completely dissolved. The pH is adjusted to 7.4 using 1N HCl or 1N NaOH and the volume adjusted to 1L.

TCT (50 ML)

50 ml PBS (without Triton –X)

0,35g Carragenin (Fluka, Sigma, Madrid, Spain)

250µl Triton X-100 (Sigma, Madrid, Spain)

25 ml of PBS are added to the remaining reagents and stirred overnight. The final volume is adjusted with the remaining 25ml of PBS.

EtOH 95% (100ml)

95ml EtOH 100%

5ml H_2O bidistilled

EtOH 75% (100ml)

75ml EtOH 100%

25ml H_2O bidistilled

EtOH 70% (100ml)

70ml EtOH 100%

30ml H_2O bidistilled

EtOH 50% (100ml)

50ml EtOH 100%

50ml H_2O bidistilled

EtOH 40% (100ml)

40ml EtOH 100%

60ml H_2O bidistilled

EtOH 25% (100ml)

25ml EtOH 100%

75ml H_2O bidistilled

EtOH 15% (100ml)

15ml EtOH 100%

85ml H_2O bidistilled

STOCK SOLUTION OF ALCIAN BLUE 8GX (100ML)

20 mg Alcian blue 8GX (Sigma, Madrid, Spain)

70 ml EtOH 100% (Merck, VWR, Lisboa, Portugal)

30ml of glacial acetic acid (Merck, VWR, Lisboa, Portugal)

KOH 1% (250ml)

2.5g KOH pellets (Sigma, Madrid, Spain)

250 ml H_2Od

STOCK SOLUTION OF ALAZARIN RED S 1% (100ML)

1g Alazarin red S (Sigma, Madrid, Spain)

100 ml KOH 1%

KOH 0.5% (250ml)

125ml KOH 1%

250 ml H_2Od

KOH 0.5% / glycerol 3:1 (100ml)

75ml KOH 0.5%

25ml glycerol (Prolabo, VWR, Lisboa, Portugal)

KOH 0.5% / glycerol 1:1 (100ml)

50ml KOH 0.5%

50ml glycerol (Prolabo, VWR, Lisboa, Portugal)

KOH 0.5% / glycerol 1:3 (100ml)

25ml KOH 0.5%

75ml glycerol (Prolabo, VWR, Lisboa, Portugal)

LB BROTH PLATES (100 ML)

Add LB Agar pellets (Sigma, Madrid, Spain) to 100 ml water and autoclave. Liquify the medium in the microwave full power aprox. 15 minutes. Let it cool to 60ºC and add ampicilin (Promega, VWR, Lisboa, Portugal) to a final concentration of 100 μg/ml followed by by 0.5mM IPTG and 80μg/ml X-Gal. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C

LB BROTH (100 ML)

Add LB pellets (Sigma, Madrid, Spain) in 100 ml water and autoclave. Add **75** μg/ml ampicilin to medium before use.

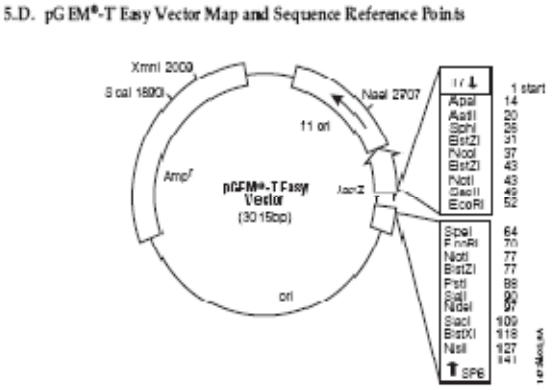

Figure A-1 Pgem-Teasy vector sequence, with identification of restriction sites. (From pGEM – T and pGEM – T Easy vector systems, Technical Manual, Promega and pBluesript II phagemid vectors, instruction manual, Stratagene).