3. METHODOLOGY

The present study consists of an integrated approach to analyze how polyploidy (diploid vs triploid) affects the development of the musculoskeletal system in rainbow trout fed diets deficient in phosphorus. In order to assess how the musculoskeletal system is modified a range of morphological, biometric, biochemical and molecular methodologies have been used. The study is based upon the hypothesis that modifications induced by triploidy may become more evident when homeostasis is challenged and for this reason diploid and triploid fish were exposed to diets supplying adequate or deficient levels of phosphorus in order to modify mineral homeostasis. In this section the main analysis conducted are organized into morphological, biometric, biochemical and molecular analysis.

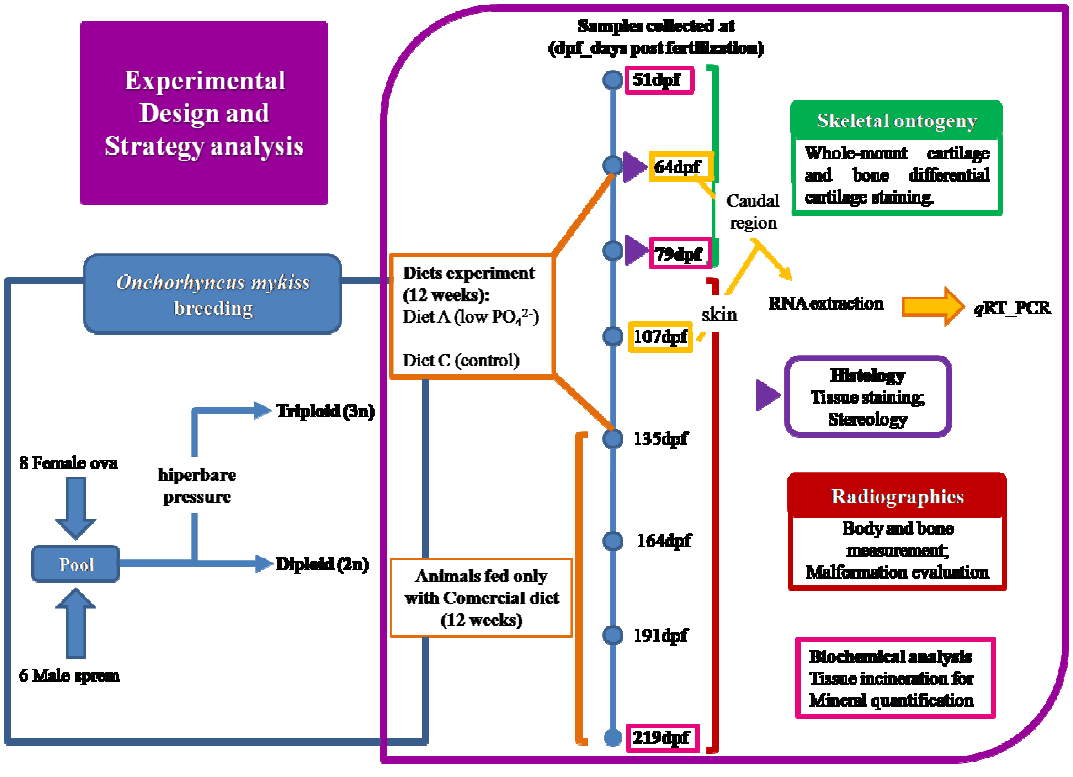

Figure 3.1 – Fluxogram presenting the experimental design and analysis performed. Fertilized eggs were produced from a pool of ova and sperm, half of the eggs were triploidized using hiperbare pressure. Samples were collected at different time points during the experiment, indicated in vertical scale bar. The experimental diet was administered at 64 dpf, stage when

the alevin started feeding and yolk resorption was complete. Two different diets were tested: Diet A with low phosphorous content (0.5% inorganic phosphorous, P) and Diet C - control (1.3 % P) for 12 weeks. After this period all juveniles were maintained for a further 12 weeks and fed commercial diet with an adequate P content. The analysis conducted consisted of: morphological evaluation including whole mount cartilage and bone staining for the first stages (—) , evaluation of skeletal ontogeny and existence of abnormalities; - Radiographies for ossified animals (—), 107 dpf - 219 dpf trout, evaluation included detection of internal abnormalities and biometry of vertebra in different body regions; Histological sections and staining (—); molecular analysis (RNA extraction and *q*RT-PCR (—)) and biochemical (calcium and phosphorous) quantification (—). All collected information was used to analyze rainbow trout skeletal ontogeny influenced by two different factors (triploid) ploidy or a limiting diet. Specific skeletal gene markers were used to assess the influence of diet on the transcriptome.

3.1. Biological trials and sampling

All the experimental rearing and respective collection of samples was carried by Dr. Stephanie Fontagné from the Institute National de la Recherché Agronomique_INRA.

3.1.1. Experimental fish and dietary trial conditions

Rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from eight females and ova fertilized using a common pool of sperm from six males in a commercial fish farm (Aqualande, Pissos, France). Half of the eggs from the common batch were triploidized by hyperbare pressure. Dead eggs and embryos were periodically removed and survival rates were expressed as percentages of the initial number of eggs used for fertilization (91.7% for diploid embryos vs. 89.6% for triploid embryos at 185 degree-days post-fertilization, dpf). After the eyeing stage, at 237 degree-days (or 21 dpf), embryos were transferred and hatched at the INRA experimental fish farm in Lées-Athas (Pyrénées-Atlantiques, France) at 7 ± 1°C. At the swim-up stage defined as day 0 of the experiment (51 dpf, 460 degree-days), fry were transferred to the INRA experimental fish farm in Donzacq (Landes, France) and randomly distributed into 12 tanks (600 larvae per 50 L fiberglass tank) supplied with spring water at 17 ± 1°C. Fish with an initial mean weight of 0.11 ± 0.01 g were hand-fed six times per day to excess or visual satiety. Each experimental diet was distributed

to 3 replicate tanks over a 12-week growth trial and a commercial diet was distributed to all groups over a further 12-week growth trial.

3.1.2. Sample collection

The final survival rate was calculated from daily mortality and from the final number of surviving fry recorded in each tank. Samples of fry were withdrawn at hatching (311 degree-days), on day 0 of the growth trial and from each tank on days 13, 28, 56, 84, 112, 140 and 168 after fasting for 24 h. Table 3.1 summarizes the age of trout sampled in days post fertilization (dpf) and describes the developmental stages. Trout were anaesthetized in diluted 2-phenoxyethanol for wet weight determination and frozen in liquid nitrogen and stored at −80°C or placed in RNAlater©, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, 10% formalin or sublimated Bouin-Hollande prior to analysis.

Table 3.1 – Age and development stage of the samples analyzed. The boxed samples correspond to those received and analyzed in the present work. dpf – days post fertilization; ** end of the feeding trial with the control and phosphorus poor diet (diet C and diet A), then feeding with a common commercial diet.

Degree days	dpf	day	Development stage
0	0	-	Fertilization
231	21	-	Eyed stage
311	35	-	Hatching
460	51	0	Swim-up stage fry
681	64	13	Fry at yolk-sac resorption
936	79	28	1 month of feeding
1412	109	56	2 month of feeding
1888	135	84	3 months of feeding**
2364	163	112	4 month of feeding
2840	191	140	5 month of feeding
3316	219	168	6 months of feeding

3.1.3. Experimental diets

Two semi-purified casein based diets were formulated (Table 3.2) to be isoproteic (53% dry matter) and isolipidic (16% dry matter). The basal diet A contained only phosphorus (P) supplied by casein, 0.45% and the control diet C was supplemented with 0.8% highly available P supplied as a 1:1 mixture of NaH_2PO_4/KH_2PO_4 resulting in 1.3% P. The commercial diet used contained 1% P.

3.1.4. Diets composition

Proximate composition of diets and fry was determined using the following procedures: dry matter was determined after drying samples at 105°C for 24 h, protein ($N \times 6.25$) by the Kjeldahl method after acid digestion, ash by incineration at 550°C for 16 h and gross energy in an adiabatic bomb calorimeter. Total lipid was extracted and measured gravimetrically according to Folch et al. (1957) using dichloromethane instead of chloroform. Total phosphorus was determined by the molybdate-blue/ascorbic acid method at 820 nm after mineralization and acid digestion (AFNOR, 1992). The specific composition of each diet applied during feeding trial is presented in table 3.2.

Table 3.2 - Formulation and composition of experimental diets (g/100g dry weight).

Diet	**A**	**C**
Ingredients		
Casein-dextrin basis[a]	82.5	82.5
Vitamin mixture[b]	5	5
Mineral mixture without P and Ca[c]	2.5	2.5
NaH_2PO_4/KH_2PO_4 (50/50)	0.00	3.36
$CaCO_3$	2.50	2.50
α-Cellulose	7.50	4.14
Proximate composition		
Dry matter (%)	91.4	93.0
Crude protein	53.8	53.2
Total lipid	16.4	16.0
Gross energy (kJ/g dry matter)	22.8	22.1
Ash	4.8	7.9
Total phosphorus	0.5	1.3

[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, 5g; menadione, 1 g; thiamin-HCl, 0.1g; riboflavin, 0.4g; D-calcium panthothenate, 2g; pyridoxine-HCl, 0.3g; cyanocobalamin, 1 mg; niacin, 1g; choline, 100g; ascorbic acid (L-ascorbyl-2-polyphosphate), 5g; folic acid, 0.1g; D-biotin, 20 mg; meso-inositol, 3 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.

3.2. Biochemical analysis

3.2.1 Calcium and phosphorus quantification

In order to establish how dietary mineral content influences skeletal composition the mineral content of easily removed bones of uniform size, the vertebrae, was determined. The vertebral column of the rainbow trout can be subdivided into 4 key anatomical regions (agrouped according to their characteristics) and three vertebrae from each region (figure 3.2) were removed based upon the shape of the vertebra, and dried at 65ºC until constant weight. This generally took 2 days and samples were weighed twice daily on a digital balance (Shimadzu AUX 120) until 3 consecutive readings were equal. Samples were then incinerated in an oven at 550ºC, for 8 hours and then 2.5 mg of ash removed from each sample and 150 µl of concentrated nitric acid (60% w/v) added and left to react for 24 h. The reaction was stopped by adding 150 µl sodium hydroxide (2M). Calcium and phosphorus were determined by spectrophotometry using a Spinreact Kit Calcium and Phosphorus-UV (Reactivos Spinreact S. A. Spain). This technique is based on the formation of a colored complex between the calcium in the sample and cresolftalein in alkaline medium. The color intensity of the obtained complex is directly proportional to the quantity of calcium present in the sample. For total phosphorus determination the technique is based on its reaction with amonium molybdate creating a phosphomolybdic complex of yellow color, and the intensity of the color formed is proportional to the concentration of inorganic phosphorus in the sample. The results are expressed as µmolCa^{2+}/mg ash or µmol Pi/mg ash.

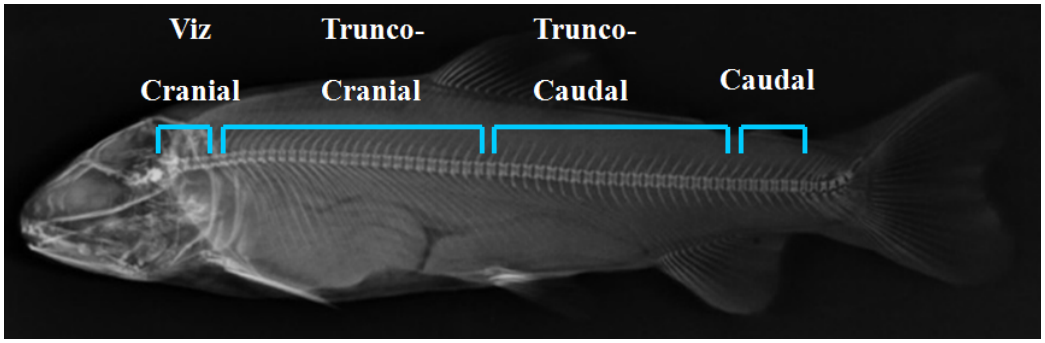

Figure 3.2 – *Oncorhynchus mykiss* radiography scheme indicating the main anatomical regions considered. Viz cranial – V1 - V8; Trunco Cranial – V 9 – V 30; Trunco caudal – V31 - V 51; and Caudal – V 52 - V 60.

Prior to calcium and phosphorus analysis in ash, optimization studies were carried out to identify the most appropriate sample dilution. The method utilized is briefly presented, 5 µl of each dilution tested (1:10, 1:25, 1:50, 1:75, 1:100) or standard was placed in a 1 ml microcentrifuge tube and 250 µl of kit reagent was added and the tubes vigorously mixed. All reagent used in the assay were warmed to room temperature (25ºC) prior to use, as the method depends on an enzymatic reaction. The optimal dilution of ash from vertebrae samples for determination of calcium and phosphorus was 1:25 Ca^{2+} and 1:75 Pi respectively. All samples were analyzed in a multiwell plate and absorbance determined using a Benchmark Biorad device. For total calcium determination the wave length utilized was 570nm, has for Phosphorus was a 340 nm.

3.3. Morphological and biometric analysis

A number of different methods are encompassed in this section and include standard biometric parameters such as, standard length, vertebral length, and analysis of whole mount stained specimens. The whole mount stained animals were used to study skeletal ontogeny and presence of malformations. For older specimens with their skeleton completely ossified radiographies were taken and evaluated.

3.3.1 Whole-mount cartilage and bone differential staining

Specimens of 51, 64 and 79 dpf age (table 3.3) were subject to whole mount cartilage and bone staining and then analyzed using a dissecting microscope in order to observe skeletal ontogeny and also to identify abnormalities. Differential staining was carried out on 15-30 individuals' simultaneously using Alcian blue 8 GX and Alizarin Red S (Faustino and Power, 1998; Taylor and Van Dyke, 1985). A synthesis of the main methodological steps is now given; specimens were washed twice (15 minutes each) in distilled water, and immersed in a staining solution of Alcian blue 8 GX (Aldrich) (0.02% in 70 % Ethanol and 30 % glacial acetic acid) for approximately 2h. The specimens were then bleached (3% H_2O_2 and 0.5% KOH) and subsequently cleared in an enzyme buffer solution composed of a solution of 30% saturated borax (dissolved in distilled water) with 0.112% (w/v) trypsin (Sigma-Aldrich) and maintained at room temperature until they became transparent. Staining of calcified tissue was carried out overnight at room temperature using an Alizarin Red S working solution composed of 25µl of stock Alizarin Red S solution (1% in 1% KOH) in 10ml KOH (0.5% - stock solution). Specimens were then washed in water to remove excess stain and passed through solutions of glycerol 3:1, 1:1 and 1:3 containing 0.5% KOH. Transfer of specimens between glycerol solutions was only carried out once they sunk to the bottom of the staining vessel. All specimens were stored in pure glycerol (Analar Normapur) at room temperature in the dark.

Table 3.3 – Number of specimens analyzed (total n=100) by alcian blue/alizarin red at different ages and fed with different diets.

Age (dph)	**Number Diploids**		**Number Triploids**	
51	10		10	
	Diet A	**Diet C**	**Diet A**	**Diet C**
64	10	10	10	10
79	10	10	10	10

Stained specimens (Figure 3.3) were observed using a stereoscopic Microscope (Olympus SZ-PT) and careful analysis of main endochondral and dermal

skeletal structures (listed in table 3.4) was carried out to establish the ontogeny of skeletal ossification. All skeletal abnormalities were noted and a photographic record of each sample was taken using a digital camera (Olympus DP11).

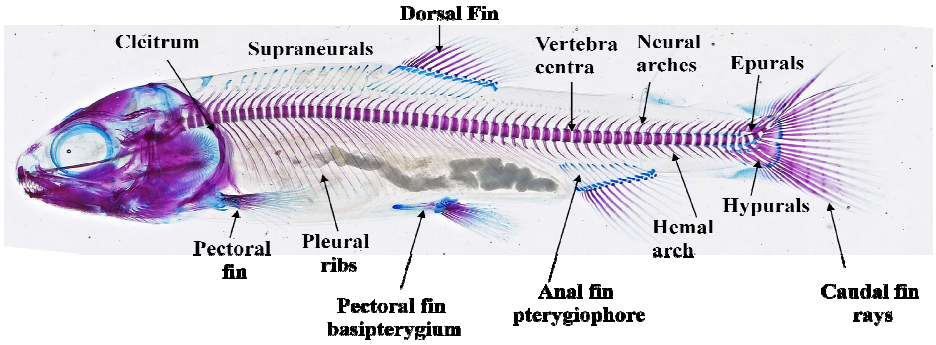

Figure 3.3- Photograph of a trout (79 dpf) stained with Alcian blue and Alizarin Red. Note the red/purple staining bone and blue staining cartilage structures. The main skeletal structures analyzed are indicated and are also listed in table 3.4. Photograph kindly provided by Dr. Stephanie Fontagné.

Table 3.4 – Skeletal structures observed in each skeletal area evaluated in order to determine skeletal ontogeny and identify abnormalities. D – Dermal structures; E – Endocondral structures.

Vertebral Column	**Pectoral Fin**	**Dorsal Fin**	**Caudal Fin**	**Supraneurals**	**Pelvic Fin**	**Anal Fin**
Pleural Ribs, **E** Vertebra Centra, **D** Neural Arches, **E** Hemal Arches, **E**	Cleitrum, **D** Actinosts , **E** Rays , **D** Coracoid , **E** Scapula , **E** Radial , **E** Postcleitrum , **D** Supracleitrum , **D**	Pterygiophores , **E** Rays, **D**	Hypurals, **E** Epurals, **E** Rays, **D** Urostyle, **D**	Pterygiophores, **E**	Basipterigium, **E** Rays , **D**	Pterygiophores, **E** Rays , **D**

3.3.2 X-rays analysis

Radiographies of trout were carried out at the *Hospital Distrital de Faro* in the radiology department using a Senographe DMR Mammography System (General Electric Medical Systems), using the manual exposure mode with 40 mAs and 40Kv. All trout specimens were subject to the same radiographic conditions and generally a batch of 15-30 samples of a similar size were radiographed simultaneously. Resulting radiographs were extracted in digital format using a FUJIFILM Computed Radiography CR Console connected to a Mammography Workstation and images, were stored as JPEG 8bit images to allow analysis on a personal computer. Digital images of the radiographed samples were analyzed using the image analysis program Image J (freeware *http://rsb.info.nih.gov/ij/ download.html*), and a range of biometric parameters were recorded.

The standard length (SL), total length (TL), the length of the head (HL) and the vertebral column length (VCL) was measured in every radiographed specimen (figure 3.4). In order to establish basic biometric parameters for the vertebral column of rainbow trout, three vertebra, at the mid-point of each of the anatomical regions identified (see figure 3.2) were measured.

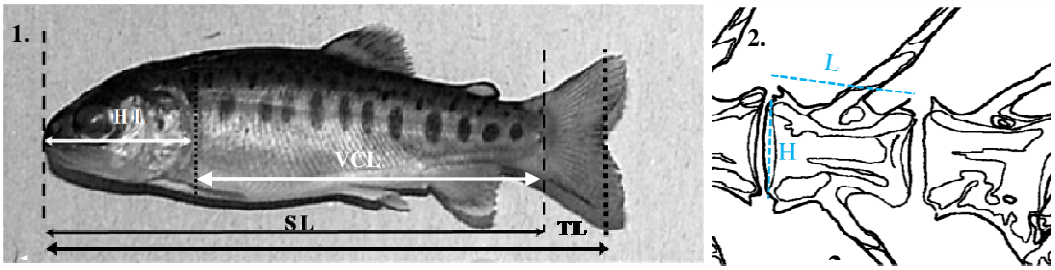

Figure 3.4 – Photograph of a rainbow trout and a schematic drawing. The parameters measured are indicated, **1.** Whole body measurements; SL Standard length, measure from the tip of the snout to the end of the caudal region; TL total length, measurement since the tip of the snout to the middle end of the caudal fin; HL head length, measured from the tip of the snout until the cleithrum. **2.** Schematic drawing of vertebra centra measurements; H – height, L – length.

Table 3.5 summarizes the number of animals radiographed and measured in each experimental group. The analysis of vertebrae height and length was previously described by Kacem et al. (2003). The vertebral area was calculated assuming that the vertebra is a cylinder and applying the formula, area=$2*\pi*(H/2)*L+2*\pi*r^2$. All the data collected were submitted to a statistical analysis using a t-student and ANOVA tests in *Sigma Stat 3.1* program.

Table 3.5 – Number of specimens radiographed and analyzed from each experimental group and at different ages in days post fertilization.

Age (dpf)	Diploid A	Diploid C	Triploid A	Triploid C
107	10	10	10	10
135	20	20	20	10
163	20	20	20	20
191	20	20	20	21
219	20	20	16	15

3.3.3 Histological and stereological analysis

3.3.3.1 Sample preparation

Material which had been fixed in paraformaldehyde and stored in methanol was used for histological analysis. The general methodology was common for all the samples and entailed an initial step of decalcification to remove calcium from tissues in order to facilitate sectioning. Decalcification was performed on trout alevin at two different developmental ages 64 dph and 79 dph. Specimens were removed from methanol and then washed 3 times for 15 minutes in DEPC water. Subsequently specimens were transferred to a 0.5M EDTA solution pH8.0 and kept in the dark at room temperature, for 7 days changing solutions every day. At the end of the decalcification period specimens were washed in DEPC water for 2 hours, changing DEPC water 3 times.

3.3.3.2 Tissue processing and wax embedding

After decalcification specimens were processed and embedded in wax for subsequent sectioning. Individual trout were cut-up into several different pieces

figure 3.5 in order to facilitate the study of specific tissue. Region 0 was used to study thyroid development, Region 1 to study the intestine, region 2 to study the muscle and region 3 to study the skin and bones.

Processing was carried out in a tissue processor (Leica TP1020) and 20-50 samples were simultaneously passed sequentially through the solution and duration described at table 3.6

Table 3.6 – Description of the solutions used, time duration and number of immersions used in the tissue processor, for tissue preparation before paraffin blocks.

Solution	Duration (minutes)	Number of immersions
Ethanol 70%	10	1
Ethanol 95%	30	2
Ethanol 100%	60	2
Ethanol : Xylene (1:1)	60	1
xylene	60	1
xylene	90	1
xylene : paraffin (1:1)	120	1
paraffin	120	1

Paraffin blocks of each of the processed regions was prepared using a hot plate incorporating a paraffin wax dispenser and a crio table (Miles Scientific).

3.3.3.3 Histological Sectioning

Serial sections (5 µm thick) were prepared from paraffin blocks of tissue using a manual microtome (Leica RM 2125). Sections were mounted on slides

previously treated with APES, using a paraffin section mounting bath (J.P. Selecta S.A.) containing DEPC water heated at 43ºC.

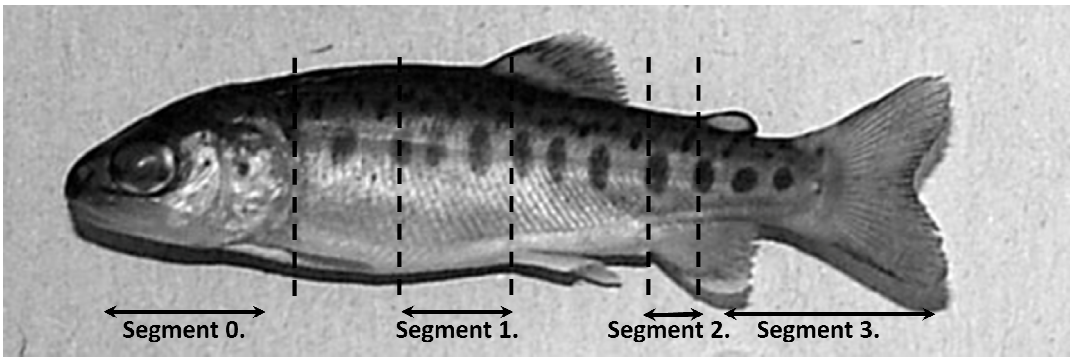

Figure 3.5 – Indication of the tissue segments used for processing and subsequent sectioning of 64 dph and 79 dph trouts.

3.3.3.4 Heamatoxylin eosin staining

In order to verify the quality and tissue organization of the sections prepared they were subject to hematoxylin & eosin staining. Sections were dewaxed, and rehydrated. In brief this entailed washing sections in two changes of xylene for 15 minutes each time. Subsequently slides were passed through a graded series of ethanol (Pronalab) two washes in 100% ethanol (5 minutes), 95% ethanol (5 minutes), 70% ethanol(5 minutes) and finally samples were washed in several changes of distilled water for 5 minutes. Tissue sections were immersed in haematoxylin for 30 seconds and then washed in tap water with a few drops of 4 M ammonia and then immersed in running tap water for several minutes to blue. This was followed by immersion in eosin Y for 30 seconds followed by a wash in distilled water with a few drops of acetic acid and then with distilled water. Sections were dehydrated through a graded ethanol series: 70%, 95% and 100%, 5 minutes each, followed by xylene (Pronalab) 2 x 15 minutes and mounted with DPX (Fluka).

3.3.3.5. Von Kossa staining

Von Kossa staining was used to specifically stain mineralized tissues. This technique demonstrates deposits of calcium or calcium salts so it is not specific for the calcium ion itself. In this method, tissue sections are treated with a silver nitrate solution and the silver is deposited by replacing the calcium and is reduced by strong light

becaming visible as metallic silver. Sections are deparaffinized in Xilol (2 x 15 minutes) followed by rehydratation trough an alcohol series (100% - 50%) and finally to water. After rinsing in distilled water, sections were incubated with 5% silver nitrate solution in a clear glass coplin jar and placed under light for 1 hour. After light exposure slides were washed three times with demineralized water, and immersed in a hypo 5% solution for 5 minutes, washed in tap water, rinsed in distilled, counterstained with Nuclear Fast Red for 5 minutes, and rinse in distilled water. Sections were dehydrate through a graded alcohol series (50% - 100%) immersed and cleared in K-clear (Kaltek srl) and a mounted using DPX (Fluka) (Sheehan D. et al 1980).

Slides were observed using a Microscope (Olympus BH2) and images captured with an Olympus digital camera DP11.

3.3.4 Muscle myotomes total counts

To determine the total number of myotomes, hematoxylin & eosin stained transverse section from segment 2 (figure 3.5) from 3 different specimens per group. Three sections from three individuals were present on each slide and sections were 5 µm thick. For analysis of myotome number slides 1, 10 and 20 were examined and the number of myotomes in each of the individuals determined in the three sections/slide (detailed in table 3.7).

Table 3.7 – Description of the treatment group and their age, tissue orientation, sections used, and section depth for determination of myotome number.

Treatment Group	**Tissue Orientation**	**Tissue depth**		
		Slide 1	Slide 10	Slide 20
DC, DA, TC, TA (Age: 64 and 79 dpf)	Transversal	15 µm	150 µm	300 µm

In each slide the total number of myotomes were determined in the left and right side of each section as illustrated in figure 3.6.

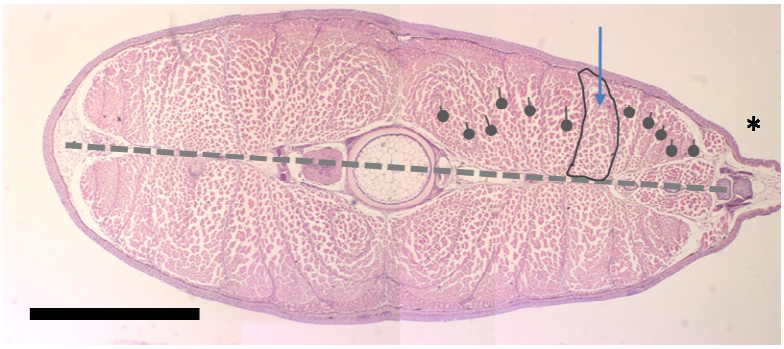

Figure 3.6 – Photograph of a typical section used to count myotomes. The number of myotomes on each section was counted on the right hand side and left hand side (the asterisk indicated the dorsal side of the trout). The arrow and the bold outline indicate the outline of a myotome, the "bold" dots indicate the myotomes visible in the upper left quadrant segments used for processing and subsequent sectioning of trout of 64 dph and 79 dph. The scale bar corresponds to 1mm.

3.4. Molecular analysis _ gene expression

To study the effect of ploidy and the dietary Phosphorus on gene expression, genes were chosen based on their importance in bone formation, namely: osteocalcin (OSC), osteonectin (OSN) and osteopontin (OSP). Two other genes, 18S and β-actin, were tested to assess their suitability for normalization and use as reference genes, to standardize for individual sample variation. The method chosen to analyse the expression of these genes was real time quantitative PCR (RT-qPCR). This method RT-qPCR is a combination of three steps: (1) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (2) the amplification of the cDNA using PCR and (3) the detection and quantification of amplified products in real time (Nolan et al 2006).

3.4.1 RNA extraction

Alevin of 64 dpf were sectioned from the anal pore to the dorsal fin and the whole caudal region of the specimen used for extractions. Samples were

homogenized in a glass homogeniser with TRI reagent (1 ml TRI for 50-100 mg tissue). Chloroform (0.2 ml /ml TRI) was added and the tubes incubated at room temperature for 15 minutes, centrifuged at 12000rpm, 4ºC for 15 minutes and the aqueous phase collected. Isopropanol was added (0.5 ml / ml TRI) to the aqueous phase and left for 2h at - 20ºC. The tubes were centrifuged using the same conditions previously indicated but for 10 minutes and the pellet retained and washed twice with ethanol 75% (with DEPC water). The pellet was then air dried for 15 minutes and dissolved in sterile water (*milli Q*, 50µl).

3.4.2 Quantification and RNA integrity analysis

The RNA concentration of each sample was determined using a spectrophotometer. A dilution (1:50) of extracted RNA was made in *milli Q* water and placed in a quartz cell. The RNA concentration was estimated after reading absorbance at two wave length: 260 nm (nucleic acid absorption peak) and 280 nm (to detect protein contamination), in a spectrophotometer (Genequant - Pharmacia).

Each RNA extract was analyzed by electrophoresis in 1.5% agarose (Lonza) gel prepared in a TBE 1x with 1% ethidium bromide (Sigma Aldrich). RNA for electrophoresis was prepared by mixing samples (10µl RNA at 1:50 dilution) with 2µl loading buffer (40 % de sucrose and 0.25 % bromophenol blue). On completion of electrophoresis gels were observed using a transiluminator with UV light (AlphaImager) and images analyzed using the program AlphaEase FC Softwear version 6.0.

To remove contaminating DNA from RNA samples a DNA-free™ Kit (Ambion) was used. Extracted RNA (2 µg/µl) was placed in a 0.5 ml tube with DNA buffer (2 µl) and DNase (1µl) and made up to a final volume of 20 µl by adding nuclease free water. The reaction was mixed gently on a benchtop vortex and incubated for 30 minutes at 37ºC. On completion of digestion DNase was inactivated by adding 2 µl of DNase Inactivation Reagent, mixing and incubating for 2 minutes at room temperature (25ºC). The DNase free RNA was harvested by centrifuging the reaction tube for 1.5 minutes 10 000xg at 4ºC and carefully removing 14 µl of the aqueous phase. The RNA was then quantified

by diluting 1:5 and measuring the sample in a Fluorometer Qubit™ (Invitrogen), with a Quant-iT™ RNA Assay Kit. In brief, a working solution was made by diluting Quant-iT™ RNA reagent 1:200 in Quant-iT™ RNA buffer and 1 µl RNA (1:5 dilution) was added. The Quant-iT™ RNA reagent (excitation/emission maxima 644/673 nm) links to the RNA and the fluorescence emitted is converted into the concentration of RNA in the sample (ng/ml).

3.4.3 cDNA synthesis and amplification

The cDNA synthesis was performed using the M-MLV reverse transcriptase procedure (Promega) and 250 ng of DNase free total RNA extracted from the caudal region of fish. 200ng random hexamers, 1 mM dNTP's and *milli-Q* water were added to 250 ng of DNase free total RNA in a reaction vial to give a total volume of 13µl and the mixture incubated for 5 minutes at 65ºC (to melt secondary structure within the template). The mixture was cooled immediately on ice for 5 minutes (to prevent secondary structure from reforming) and 4 µl M-MLV 5x Reaction buffer, 2 µl water, 0.5 µl Ribonuclease inhibitor (RNasin), and 0.5 µl M-MLV reverse transcriptase added to give a final volume of 20 µl. A negative control reaction was carried out in which reverse transcriptase was excluded. The cDNA synthesis was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) with the following program: 10 minutes at 20ºC following 50 minutes at 42ºC, 5minutes at 72ºC.

To confirm the efficiency of cDNA synthesis 18s ribosomal RNA was amplified in all samples using a standard RT-PCR procedure. In brief, 1µl of template cDNA was mixed with a reaction mixture containing 2.5 µl reaction buffer (EuroTaq - Euroclone), 0.75 µl 50mM $MgCl_2$ (EuroTaq - Euroclone), 0.3 µl dNTPs, 1 µl of 10pmol/µl primers (forward and reverse), 0.1 µl Taq polymerase (EuroTaq - Euroclone). *Milli Q* water was added to perform a reaction volume of 25 µl for each cDNA. PCR reaction the following thermocycle was used: 2 minutes at 95ºC; 25 cycles of 45 seconds at 95ºC, 30 seconds at 59ºC and 45 seconds at 72ºC; a final cycle of 2 minutes at 72ºC completed the cycle.

3.4.4 Real Time Reverse Transcription Polimerase Chain reaction (qRT-PCR)

3.4.4.1 General Technique

The traditional PCR technique measures the amount of product at the end point of amplification, whilst the *q*PCR technique measures the amount of PCR product after each round of amplification. *q*PCR technique measures the amplification products as they are produced, using a fluorescent label. As the amplified products are formed, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, and the fluorescence values are recorded at each amplification cycle and reflect accumulating DNA molecules. The concentration of amplified product (cDNA) is directly proportional to the fluorescence signal and a linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. The point at which detected fluorescence is significantly greater than the baseline (or background) is called the threshold cycle or Ct Value. The Ct Value is the most important parameter for quantitative PCR. This threshold is established to quantify the amount of DNA in the samples. It is inversely correlated to the logarithm of the initial copy number. The threshold should be set above the amplification baseline and within the exponential increase phase (which looks linear in the log phase). Most instruments automatically calculate the threshold level of fluorescence signal by determining the baseline (background) average signal and setting a threshold 10-fold higher than this average. In theory, an equal number of molecules are present in all of the reactions at any given fluorescence level. Therefore, at the threshold level, it is assumed that all reactions contain an equal number of specific amplicons. The higher the initial amount of sample DNA, the sooner the accumulated product is detected in the fluorescence plot, and the lower the Ct value (Sigma-Aldrich, 2008).

3.4.4.2 Genes of interest

RT-qPCR protocols had been developed in a previous study and so it was unnecessary to carry out primer development and optimization of the specific PCRs. The method for quantification was based on the standard curve method and specific genes measure were osteocalcin, osteonectin and osteopontin primers and amplicon size are indicated in table 3.8.

3.4.4.3 Real-Time PCR preparation and pre-run tests

Before qPCR analysis was performed on the samples it was necessary to run test reaction to verify the standards and also to establish the appropriate sample dilution in the reactions. Table 3.8 indicates the results of the optimization and the conditions for the Real-time PCR.

Table 3.8 – q PCR primer designation, sequence, annealing temperature (Ta), cDNA dilution used and size of the reaction product in base pairs.

Gene Primers name	Primer	Ta	cDNA dilution used	Size bp
NS_OSC_FW	TCAACCCACACCACTTAC	61	1:5	121
NS_OSC_RV	CTCTCCCAGAAACACCGATCG			
NS_OSN_FW	GCAGGTAGAGACTGGAGACAAGC	58	1:5	142
NS_OSN_RV	TTCCACCTCACACACCAAAAGG			
NS_OSP_FW	ACAGCAGCAACAGTAGTGAGCTTC	59	1:5	119
NS_OSP_RV	GGAGCATCGGTAGTAATGGTTCGG			

3.4.4.4 Real-Time PCR

After optimization of the *qPCR* conditions and standard curve these were applied to the experimental samples analysis.

For each gene reaction 2µl of an appropriate dilution of cDNA (table 3.8) was added to 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Portugal), and forward and reverse primers to a final concentration of 100 nM

added and the final volume adjusted to 20 µL with sterile water. The PCR reaction took place in a sealed 96 multiwell plate (Biorad, Portugal) in a thermocycler iCycler (Biorad, Portugal). The reaction was initiated by activation of Taq DNA polymerase by heating at 95ºC during 10 minutes, and followed by 50 cycles composed of a denaturing step at 95ºC during 30 sec and an annealing step for 30 s at the optimum temperature determined for primer pairs of target templates (table 3.8) and an elongation step at 72ºC for 30 s. The reaction fluorescence was measured in each cycle after the extension step using an iCycler iQ Real-Time Optical Detection System (Bio-Rad, Portugal). After the thermocycling reaction, a melting curve of the obtained product was made by heating, starting at 65ºC and with a rate 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.

3.4.4.5 Data Processing

The results from *qPCR* was acquired using iCycler iQ Optical System Software Version3.1 (Bio-Rad, Amadora, Portugal). This software allows the input of known concentrations of the standards and after manual calibration of threshold position above background fluorescence, by the user, it automatically calculates the efficiency, the correlation coefficient and the starting quantities of the gene in study.

3.4.4.6 Data Normalization

For the normalization of gene expression data in absolute quantification is used to correct sample-to-sample variation that could have arisen for example due to the amount of tissue extracted or cDNA synthesis efficiency. The ideal control gene should be expressed in an constant manner regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment (Schmittgen and Zakrajsek, 2000). For the normalization the selected gene was the beta actin, because it was the one that presented no significant differences between treatments.

3.5. Statistical analysis

Values are shown as mean ± SEM unless otherwise stated. The statistical analysis was performed using the program *Sigmastat 3.1*. Differences between groups were established by ANOVA tests or t-student (in *Sigma Stat 3.1*) after assessing normality and homogeneity of variances. Groups were considered significantly different at $p < 0.05$.