



UNIVERSIDADE DO ALGARVE
Faculdade de Engenharia de Recursos Naturais

Biotechnology in Aquaculture: polyploidy versus transgenic technology

Dissertação de Mestrado Integrado em Engenharia Biológica

Ângela Alexandra Martinho Ramos

Faro 2009



UNIVERSIDADE DO ALGARVE
Faculdade de Engenharia de
Recursos Naturais



Biotechnology in Aquaculture: polyploidy versus transgenic technology



Dissertação de Mestrado Integrado em Engenharia Biológica

Ângela Alexandra Martinho Ramos

Orientadora: Prof Dr. Deborah M. Power

2009

Declaração: O conteúdo, e execução do trabalho experimental e interpretação de resultados é da exclusiva responsabilidade da autora

Ângela Alexandra Martinho Ramos

ACKNOWLEDGMENTS

I would like to acknowledge and give a special thanks to my supervisor, Professor Deborah Power, for her patience throughout this long process and above all for her enormous support with this work, thank you very much for all Professor. It is a privilege to learn with you!

I also would like to thank Professor Adelino Canário for accepting me in his team.

To Dr. Stephanie Fontagné a special thanks, because she is the responsible for providing all of the samples and also for resolving some doubts concerning the experimental trial and also for the encouragement and understanding.

A special thanks to Dr. António Abrantes for all the x-rays, and for providing knowledge in a different study area. Also a special acknowledgment to Dr. Patricia Pinto, who tutored me on the quantitative real time qPCR technique, to Alexandra Filipe, Dr. Pedro Guerreiro, Elsa Couto, Teresa Sancho and Mar Huertas for helping me in some parts of this work.

For all the people in the CME group a big thanks, in special for Nádía Silva (that shared most of this work) and Rita Costa that accompanied me and supported me throughout all of this process, and above all for your friendship. Also very special thanks to Silvia Gregório for her precious help with the accomplishment of innumerable tasks allowing an increase in rhythm of work. To all the people in lab 3.32 I am very pleased to work with you all.

To some special friends that were available to listen and support in innumerable situations to Dulce Estevão, Anabela Nobre, Ana Evaristo, Monika Grunner and Madalena Bentes, you are all very special to me and thanks for all. Nonetheless acknowledge Mestre Jorge Veiga e Castro for the friendship and the permanent scientific encouragement.

To Leonel a special appreciation for all the love and also for believing in my challenges, and for all support in every shared moment throughout this thesis. And finally I would like to thank my family, my parents and sister, that give always a tremendous strength giving a renewal power, and for all their love and support in every moment of my life.

LIST OF ABBREVIATIONS

CA – Caudal

Ca – Calcium

DA – Diploid diet A

DC – Diploid diet C

GH – Growth Hormone

GMO's – Genetic modified organisms

OSC - Osteocalcin

OSN - Osteonectin

OSP- Osteopontin

P- Phosphorous

TA – Triploid diet A

TC – Triploid diet C

TCR-Trunco Cranial

TCA – Trunco caudal

VCR – Vizcero cranial

ABSTRACT

Triploidy is the condition in which somatic cells contain three sets of chromosomes which might be a consequence of environmental changes or hybrid stabilization, though in the present work the ploidy induction was done by hyperbare pressure. Diploid and triploid Rainbow trout, *Onchorynchus mykiss* (Walbaum, 1792) were the species characterized in this thesis. Besides ploidy a modification in the mineral availability of phosphorous, was applied influencing the whole body mineral homeostasis in diploid and triploid trout.

The skeletogenesis in triploids animal is different from the diploid, having a general delay, the mineral deficient diet has also an impact on the ossification retreating. It allowed studying the development through triploids, which at the end of this trial they grew less than diploids. Vertebra area is also affected varying according to their regions, total number of vertebra differs, and in average triploids have less one vertebra than diploid groups. And there are also histological differences at a muscle level, having triploid Diet A less myotomes in average.

Animals present a high capacity in recovering, once they start eating a normal diet in mineral composition, they restore their mineral content in bone structures in some situation an over calcification is observed (Diploid A). In a molecular level, there were any molecular differences observed only a high variability at the OSN levels.

Key words: Diploids, Triploids, Skeletogenesis, Ontogeny, mineral content.

RESUMO

Triplóidia é um estado, em que as células somáticas contém três conjuntos de cromossomas (3n). Pode ocorrer naturalmente ou ser induzido experimentalmente com este estudo em que foi usada uma câmara hiperbárica. A espécie modelo utilizada foi a truta (*Onchorynchus mykiss* Walbaum, 1792) Diploídes e Triplóides. Para além da manipulação na ploidia efectuou-se uma alteração na disponibilidade do mineral fósforo e foi avaliado o seu efeito no desenvolvimento do sistema musculo-esquelético, usando parâmetros morfométricos, biométricos, bioquímicos e moleculares.

Verifica-se uma diferença na esqueletogénese em trutas triplóides comparativamente às diploídes, denotando-se um atraso na ossificação em ambos os grupos triplóides (dieta controlo e dieta restrita em fósforo). A acumulação corporal total de cálcio em ambos os grupos diploides e triploides de alevinos alimentados com dieta pobre em fósforo, é significativamente mais baixa ($P < 0,05$) comparativamente a alevinos diploídes ou triplóides alimentados com dieta controlo. Constata-se uma alteração significativa nas vertebrae de trutas cujo esqueleto estava completamente formado, este efeito varia consoante a região da coluna vertebral. De um modo geral trutas triploides têm menos vertebrae. Nas quatro regiões identificadas e estudadas da coluna vertebral, encontraram-se diferenças significativas ($p < 0,01-0,001$) na área das vertebrae. Os marcadores moleculares avaliados por *PCR* em tempo real, Osteocalcina, Osteonectina e Osteopontina mostraram baixa variabilidade entre nos indivíduos analisados, e não se obtiveram diferenças conclusivas entre os grupos experimentais a 64 dpf de idade.

Em conclusão, a triploidização afecta o esqueleto de truta. Um fornecimento deficiente em fósforo afecta significativamente a ontogenia esquelética, morfometria e parâmetros bioquímicos de trutas diploídes e triplóides.

Palavras Chave: Diploíde, Triplóide, esqueletogénese, conteúdo mineral.

TABLE OF CONTENTS

1. Introduction.....	1
1.1. Development.....	1
1.1.1.General overview.....	1
1.1.2.Ectoderm,endoderm, and mesoderme.....	2
1.2. Skeletal System.....	3
1.2.1 Skeleton origin and function.....	3
1.2.2. Skeletal tissue metabolism.....	4
1.2.3. Skeletal tissue composition.....	5
1.2.4. Skeletal cell differentiation.....	7
1.2.5. Skeletal tissue endocrine regulation.....	8
1.3. Muscle.....	9
1.3.1. General.....	9
1.3.2. Organization of muscle structure.....	11
1.4. Mineral requirement.....	12
1.4.1. Calcium and phosphorus.....	12
1.4.2. Phosphorus deficiency deformities.....	12
1.5. Biotechnology applied to aquaculture and lifesciences.....	13
1.5.1. Ploidy vs Transgenics.....	14
1.5.2. Triploid fish production.....	15
1.5.3. Morpho-anatomical changes in triploids.....	17
1.5.4. Growth performances in triploids.....	18

1.6. The experimental model - Rainbow trout.....	19
2. Thesis Objectives.....	20
3. Methodology.....	21
3.1. Biological trials and sampling.....	22
3.1.1. Experimental fish and dietary trial conditions.....	22
3.1.2. Sample collection.....	23
3.1.3. Experimental diets.....	24
3.1.4. Diets composition.....	24
3.2. Biochemical analysis.....	26
3.2.1 Calcium and phosphorus quantification.....	26
3.3. Morphological and biometric analysis.....	27
3.3.1 Whole-mount cartilage and bone differential staining.....	28
3.3.2 X-rays analysis.....	30
3.3.3 Histological and stereological analysis.....	31
3.3.3.1 Sample preparation.....	31
3.3.3.2 Tissue processing and wax embedding.....	31
3.3.3.3 Histological sectioning.....	32
3.3.3.4 Heamatoxilyn eosin staining.....	33
3.3.3.5 Von kossa staining.....	33
3.3.4 Muscle myotomes total counts.....	34
3.4. Molecular Analysis- gene expression.....	35
3.4.1 RNA extraction.....	36
3.4.2 Quantification and RNA integrity analysis.....	36

3.4.3 cDNA synthesis and amplification.....	37
3.4.4 Real Time Reverse Transcription Polimerase Chain reaction (qRT-PCR).....	38
3.4.4.1 General Technique.....	38
3.4.4.2 Genes of interest.....	39
3.4.4.3 Real-Time PCR preparation and pre-run tests.....	39
3.4.4.4 Real-Time PCR.....	40
3.4.4.5 Data Processing.....	40
3.4.4.6 Data Normalization.....	40
3.5. Statistical analysis.....	41
4. Results.....	42
4.1 Survival rate.....	42
4.2 Biochemical analysis.....	42
4.2.1 Whole alevin Ca and P content.....	42
4.2.2 Body region calcium and phosphorous quantification results.....	44
4.3 Morphological and Biometric Analysis.....	46
4.3.1. Ontogenic Evolution.....	46
4.3.2 X-rays analysis.....	52
4.4 Histology - muscle myotome total counts.....	58
4.5 Molecular Analysis- Gene expression.....	60
4.5.1 β -actin.....	60
4.5.2 Osteocalcin.....	61

4.5.3 Osteonectin.....	62
4.5.4 Osteopontin.....	62
5. Discussion.....	64
6. Final conclusions.....	70
7. Future work.....	71
8. References.....	72

LIST OF FIGURES

Figure 1.1 _ Schematic representation of four vertebrate model organisms.....	2
Figure 1.2 - Overview of zebrafish embryogenesis.....	10
Figure 1.3 – General representation of the process of triploidy induction.....	17
Figure 1.4 – Schematic presentation and photograph of <i>Onchorynchus mykiss</i>	19
Figure 3.1 – Fluxogram representing the experimental design and analysis performed....	21
Figure 3.2 – <i>Oncorhynchus mykiss</i> radiography scheme.....	27
Figure 3.3 - Photograph of a trout (79 dpf) stained with Alcian Blue and Alizarin Red...	29
Figure 3.4 – Photograph of a rainbow trout and a schematic drawing.....	31
Figure 3.5 – Schematic representation of the tissue segments.....	33
Figure 3.6 – Photograph of a typical section used to count myotomes.....	35
Figure 4.1 –Survival rate (%) in each experimental group during the feeding trial	40
Figure 4.2 – Determination of whole body calcium (A) and phosphorus (B).....	40

Figure 4.3 –Radiography of a rainbow trout diploid control of 219 dpf.....	44
Figure 4.4 – A. Total mean Ca concentration ($\mu\text{molCa}^{2+}/\text{mg Ash}$) and the SEM.....	45
Figure 4.5 - Diagram representing the ontogenic development of rainbow trout skeleton.....	47
Figure 4.6 – Cumulative counts \pm SEM, sum of all analyzed.....	48
Figure 4.7 – Cumulative counts \pm SEM, sum of all analyzed structures.....	49
Figure 4.8 - Total number of vertebra counted in individuals.....	51
Figure 4.9 – Mean area \pm SEM (cm^2) of vertebra from each vertebral region.....	52
Figure 4.10 – Standard length measurements mean (cm^2).....	55
Figure 4.11 – Ratio of head length: body length presented as mean \pm SEM (cm^2)	56
Figure 4.12 – Representation of the Ct value for the sample expression of β -actin.....	59
Figure 4.13 – Logarithm of the ratio of the square means of the expression of OSC.....	60

Figure 4.14 – Logarithm of the ratio of the square means of the expression of OSN.....61

Figure 4.15 – Logarithm of the ratio of the square means of the expression of OSP.....62

LIST OF TABLES

Table 1.1 – Components of mammalian bone.....6

Table 1.2 – Description of methods applied for inducing triploidy.....16

Table 3.1 – Age and development stage of the biological samples analyzed.....23

Table 3.2 - Formulation and composition of experimental diets (g/100g dry weight).....25

Table 3.3 – Number of specimens analyzed (n=100) by alcian blue/alizarin red S28

Table 3.4 – Skeletal structures observed in each skeletal area evaluated.....29

Table 3.5 – Number of specimens.....31

Table 3.6 – Description of the solutions used the duration and the number.....32

Table 3.7 – Description of the treatment group and their age.....37

Table 3.8 – Q PCR primer designation, sequence, annealing temperature (Ta).....	39
Table 4.1 - Results of the statistical analysis applied	43
Table 4.2 – Results of the t-student test results and each hypothesis tested.....	46
Table 4.3 – Results presented for the endochondral and dermal ossification.....	51
Table 4.4 - Results of t-student and each hypothesis tested for vertebra area	55
Table 4.5 - Summary table with the t-student test results and each hypothesis.....	57
Table 4.6 – Mean, standard deviation, standard error of the mean (SEM)	58
Table 4.7 – CT values results, mean value Standard error and SEM.....	60