UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

NUTRITIONAL GENOMICS STUDY: GENE EXPRESSION AND VALIDATION OF SNP (SINGLE NUCLEOTIDE POLYMORPHISM) IN CANDIDATE GENES ASSOCIATED WITH GROWTH IN ZEBRAFISH (*Danio rerio*) FED PLANT PROTEIN-BASED DIET.

Doctoral Thesis
In Fulfillment of the
Requirements for the Degree
Doctor of Sciences in Natural Resources
by

PILAR ELIZABETH ULLOA HUILCAMAN

TEMUCO – CHILE 2012

NUTRITIONAL GENOMICS STUDY: GENE EXPRESSION AND VALIDATION OF SNP (SINGLE NUCLEOTIDE POLYMORPHISM) IN CANDIDATE GENES ASSOCIATED WITH GROWTH IN ZEBRAFISH (*Danio rerio*) FED PLANT PROTEIN-BASED DIET.

Esta tesis fue realizada bajo la supervisión del Director de Tesis, Dr. ROBERTO FERNANDO NEIRA ROA, Departamento de Producción Animal, Universidad de Chile y por la Co-Directora de Tesis, Dra. Patricia Iturra Constant, Programa de Genética Humana, Universidad de Chile y ha sido aprobada por los miembros de la comisión examinadora

PILAR ELIZABETH ULLOA HUILCAMAN

……..…..……

DIRECTOR PROGRAMA
DOCTORADO EN CIENCIAS DE
RECURSOS NATURALES

……

Dr. ROBERTO NEIRA ROA

…….....

Dra. PATRICIA ITURRA CONSTANT

……

DIRECTOR PROGRAMA
UNIVERSIDAD DE LA FRONTERA

……

Dr. MIGUEL ALLENDE CONELLY

……

Dra. CAROLINA SHENE DE VIDTS

……

Dra. ANA GUTIERREZ MORAGA

……

Dr. GONZALO GAJARDO GALVEZ

¿Dónde estabas tú cuando yo fundaba la tierra?
Házmelo saber, si tienes inteligencia.

¿Quién ordeno sus medidas, si lo sabes?
¿O quién extendió sobre ella cordel?

¿Sobre qué están fundadas sus bases?
O quien puso su piedra angular,

Cuando alababan todas las estrellas del alba,
Y se regocijaban todos los hijos de Dios?...

Job 38:4-7

-Quisiera pensar que la Ciencia vaya confirmando la Fé y que a la Ciencia siempre le falten respuestas para que no desvanezca la Fé-.

Dedico esta tesis a mi querida familia,

Mis padres: Edgardo & Elizabeth; Mis hermanos: Areli & Boris; mi pequeño sobrino Brunito y mi amor Alex.

Agradecimientos

Quisiera agradecer profundamente a Dios por darme la vida y salud para terminar con éxito esta tesis doctoral.

Agradezco en forma muy especial a mí querida Familia quienes han sido mi mayor soporte emocional: A mi mamá Elizabeth quien ha sido siempre un ejemplo de admiración, esfuerzo y valentía. A mi papá Edgardo por sus consejos alentadores en momentos difíciles, a mis hermanos Boris y Areli por su incondicional amor, apoyo y amistad.

Agradezco a mis tutores de tesis, profesores Roberto Neira y Patricia Iturra quienes me brindaron su apoyo y confianza desde el primer momento para desarrollar esta tesis doctoral. Muchas gracias por sus valiosos aportes y críticas revisiones de mi trabajo.

Agradezco al Dr. Cristian Araneda por la ayuda en el desarrollo experimental de esta tesis y unidad de investigación cuando me estaba introduciendo al mundo de los peces. Agradezco también su amistad y apoyo en muchos momentos.

Agradezco a la Directora del Programa en Ciencias de Recursos Naturales Dra. María de la Luz Mora por su constante apoyo y confianza para darme la libertad en desarrollar esta tesis en la Universidad de Chile y Universidad de California-Davis, Estados Unidos.

Quisiera agradecer al grupo de trabajo del Laboratorio de Biotecnología en Acuicultura de la Universidad de Chile y a mis compañeros y amigos de estudio. Agradezco a María Hilda Avellanal, Jean Paul Lhorente, María Eugenia López, María Eugenia Larraín, Herne Castillo y Dinka Mandacovich. Agradezco en forma especial a Claudio Pérez por su valiosa ayuda en las técnicas de laboratorio y a Carla Lizama con quien compartí largas horas de trabajo.

Quiero agradecer al Dr. Miguel Allende por facilitarme peces cebra los cuales fueron usados para generar la población de peces usados en esta tesis. Agradezco también a Catalina Lafourcade por las valiosas recomendaciones en la crianza de peces.

Agradezco a la Universidad Católica de Temuco, Departamento de Acuicultura, en especial al Dr. Aliro Bórquez por facilitarme todos los instrumentos necesarios para la fabricación de las dietas experimentales. Agradezco a Dr. Stephen Gunter por generar la formulación nutricional de las dietas y en forma especial a Alex Cabrera por su valioso aporte en la extrusión de las dietas.

Agradezco en forma especial a la Dra. Natalia Lam, Dr. Jurij Wacik y Andrea Bravo por la ayuda brindada en la recolección de muestras de peces (musculo y aleta). Gracias por su tiempo y trabajo durante los largos días de muestreo.

Quisiera agradecer a la Dra. Andrea Peña de la Pontificia Universidad Católica de Valparaíso por su admirable solidaridad y profesionalismo, así como su excelente disposición para brindarme su ayuda en la técnica de PCR en tiempo real. Muchas Gracias.

Quisiera agradecer a la Universidad de California-Davis, Departamento de Animal Science y en especial al grupo de trabajo del Laboratorio de Genómica Animal. Agradezco profundamente al Dr. Medrano por darme la oportunidad de estar un año en su laboratorio aprendiendo de la técnica de RNA-sequencing. Agradezco su gran ayuda, consejos y críticos aportes en mi investigación. Quiero agradecer también a Alma Isla-Tejos por su generosa amistad, así como sus valiosos aportes en las técnicas de laboratorio. Al Dr. Gonzalo Rincón por su excelente voluntad en enseñarme el uso de herramientas bioinformáticas para poder interpretar los resultados de mi trabajo. A Rashida Lathan por su amistad, por compartir aquellas tardes de arduo trabajo, así como tardes de películas y preparar esa exquisita torta de zanahoria. A Rodrigo Gularte por su acogida en el laboratorio y ayudarme a construir algunas figuras en los resultados de mi tesis. A Saumya Yapa por su buena onda siempre. A Cinzia Ribecca, Pablo Peraza, Nicolas Grasso y Eva Ceh estudiantes de intercambio de diferentes países con quienes compartimos gratos momentos de trabajo y camaradería.

Agradezco a mis amigos Johana Kreither & Javier López-Calderon por su entrega, confianza y amistad durante mi estadía en los Estados Unidos. Fueron una linda familia para mí.

Agradezco a mis amigos y compañeros del doctorado: Loreto Manosalva, Andrea Silva, Elizabeth Garrido, Cecilia Paredes, Paula Aguilera, Karen Manquian, Sebastián Meier, Sebastian Elgueta, Marcelo Panichini, Raúl Orrego, Emilio Jorquera, Claudio Inostroza, Edgardo Cruces y Oscar Martínez. En forma especial quiero agradecer a mi gran amiga Rayen Millaleo por su incondicional amistad y valioso apoyo en todos los momentos. Gracias Rayen!!

Agradezco al Consorcio Empresarial y Biotecnológico Aquainnovo S.A. por financiar mis estudios durante cuatro años, así como parte del desarrollo de la tesis doctoral. Agradezco a la Beca de Apoyo de Tesis Conicyt AT-20041052, Becas de pasantía en el extranjero Becas-Chile 2401230, Beca Término de Tesis Doctoral Conicyt TT-21, Beca de Asistencia a Reuniones Científicas Conicyt. Agradezco también el apoyo económico del Programa Evoluciona de la Universidad de La Frontera, y al Programa de Doctorado en Ciencias de Recursos Naturales por financiar la presentación a diferentes congresos nacionales e internacionales.

Outline Thesis

Aquaculture is the fastest growing food-producing area in the world. Between 1970 and 2010, the aquaculture sector has maintained a current average growth rate of 8.8 percent per year. However, world capture fisheries to produce fish meal and fish oil, that constitute the highest protein and lipid sources available within the animal feed (fish, chicken, bovine) have shown a trend to decline in the last decades. This situation, along with the increased demand resulting from rapid growth of the aquaculture sector has generated the high cost in fish meal. Feed in farmed fish represents about 40-50 percent of the production costs at farm level and about 30-35 percent of the total cost of the final product. Limited availability of fish meal and concerns regarding economic and environmental sustainability has driven the farmed fish industry to look for alternative protein sources. Progress has been made on the replacement of fish meal with plant protein sources, including barley, canola, corn, cottonseed, peas, lupines, soybeans and wheat.

Considering that in the future the aquaculture activity will depend strongly on the alternative of plant protein in the diet, it should be important to identify genetic differences among individuals in response to these new formulated diets. In light of this scenario arises the question: How do fish respond genetically (gene expression and genetic polymorphisms) and phenotypically (growth) when they are fed with a plant protein-based diet?

This question was addressed in this thesis through studies of "nutritional genomics" which integrates nutrition and genetics on two approaches: Nutrigenomics and Nutrigenetics. Nutrigenomics focuses on studying how diet affects the expression of certain genes. Nutrigenetics refers to how specific genetic variants influence the phenotype (growth) in response to the diet. These genetic variants often occur as single nucleotide polymorphisms (SNP) in coding genes.

Growth has been for years the main objective of selection in breeding programs in fish. Currently, genes associated with growth have been mapped and sequenced becoming to be candidate genes to investigate differences in gene expression and allelic variants associated to this phenotype. The candidate genes are chosen according to the information about their role in metabolic pathways, and whose protein products are contributing to the phenotype.

The change in fish nutrition is one relevant factor that affects multiple physiological functions, which are reflected in important production traits such as growth. There is limited research that focuses on studying how plant protein diet affects the gene expression related to growth in relation to an animal protein diet.

Besides there is no information on the comparison in the expression of these kinds of genes among individuals of different growth rates, neither knowledge about genetic variants (SNP) associate with fish growth in response to new plant formulated diets.

In this study, zebrafish has been used as a model organism due to the fact that they possess advantages in relation to farmed fish. They have small size, short generation time (12–14 weeks) and they are classifying as omnivores, they can eat a great variety of foods including animal and plant protein diets. In addition, this species have available a larger amount of genomics information important for genetics studies.

This thesis aimed to examine gene expression in candidate genes related to growth in zebrafish and identify SNP in differentially expressed genes among adult fish of different growth rates in response to plant protein-based diet. For achieving this, a series of assays were carried out, which are presented in the following chapters:

The first chapter is the Introduction, which provides the theoretical background, hypothesis, general and specific objectives. The second chapter: *"Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquaculture fish"* corresponds to a review, which provides theoretical background that support this research.

The third chapter: "*Growth response and expression of muscle growth-related candidate genes in adult zebrafish (Danio rerio) fed plant and fishmeal protein-based diets"*. The main objective of this study was to examine growth response and evaluate the expression of growth-related genes in the muscle of adult zebrafish, from a population of 24 experimental families fed with plant and fishmeal protein-based diets. To control for familiar variation, each family was split to create two fish populations with similar genetic backgrounds. The fish were fed from 35 at 98 days post fertilization (dpf) with fish meal (FM, control diet) and plant protein (PP, experimental diet) as unique protein source in their diets. Measurements were taken to evaluate growth response to each diet. In order to examine the PP diet's effect on gene expression, individuals from three similar families with average growth in both populations were selected. In order to examine the effect of familiar variation, the three families were evaluated separately. At 98 dpf, eight growth-related genes (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Myostatin1b*) were evaluated in males and females. This study demonstrated that PP diet reduce fish growth as compared with FM diet in both sexes. In males, *Myogenin*, *Mrf4* and *Igf2a* showed changes attributable to the PP diet. In females, the effect of the PP diet did not modulate the expression in any of the eight genes

studied. The family variation effect on gene expression was observed in males and females among families. This study showed that plant protein and family variation have important effects on gene expression in fish muscle.

The fourth chapter: *"Candidate gene expression in muscle of adult zebrafish with lower and higher growth in response to plant protein-based diet"*. In this chapter, we examine the gene expression related to growth in muscle of lower and higher growth zebrafish in response to plant protein-based diet. A population of 24 experimental families was fed with a balanced plant protein diet (57.8 % digestible protein, 7.1 % digestible lipids, and 391cal/kg-1 DM energy) from 35 to 98 dpf. Measurements were taken to evaluate growth response to a diet. From 440 males and 339 females, five percent in both ends of the normal distribution for weight gain were selected in each case (n~17). From this individuals eight lower growth fish (average weight = 52 mg in males and 64 mg in females) and eight higher growth fish (average weight = 228 mg in males and 294 mg in females) were used to gene expression analysis. Eight genes (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Myostatin1b*) were evaluated in muscle of males and females. In males with higher growth, *Myogenin* was under-expressed (X0.31 ± 0.08) as compared to lower growth, whereas *Pld1a* was over-expressed (X1.31 ± 0.08). In females with higher growth, *Myostatin1b* (X1.82 ± 0.31) and *mTOR* (X1.55 ± 0.27) were over-expressed as compared to lower growth. This study demonstrated that differential gene expression pattern in fish muscle between lower and higher growth could give account of different genetic background that underlies the growth phenotype. This information allows us to identify those genes involved in growth, which are modulated by plant nutrients in males and females.

The fifth chapter: *Identification of SNP in differentially expressed genes between lower and higher growth zebrafish fed with plant protein-based diet using RNA-seq and growth association study*. The aim of this study was to identify SNP in differentially expressed genes in muscle of lower and higher growth zebrafish fed with a plant protein-based diet, and performing a growth association study. Results demonstrated in average a total of 17,227 genes expressed in fish muscle. From these genes, 124 genes were differentially expressed between phenotypes, 54 of them were over-expressed in lower growth fish and 70 genes were over-expressed in higher growth fish. A total of 165 SNP were selected from these genes and were used to genotype 240 fish for association study. Association analysis revealed five SNP associated with growth, each one of them was located in *Nars, Acta1b, Plac8l1, OU-44, and Lmod2b* respectively. The functions of these genes are related to skeletal muscle formation. Two allelic variants were localized in exons and correspond as silent mutations. The other three variants were localized in UTR sites which could give account of differential gene expression. These SNP could be molecular

markers in disequilibrium linkage with other genes or QTL that underlies to the phenotype. These allelic variants can be identifying in farmed fish through comparative genomics.

The sixth chapter corresponds to the general discussion and the seventh chapter corresponds to the conclusions and future perspectives.

Table of contents Pag.

Table Index

Figure Index

Chapter 1. General Introduction

1. Introduction

1.1. World Aquaculture

In the last three decades, world production of farmed fish has grown at an average rate of 8.8 percent per year (FAO, 2012). In 2010, global aquaculture production reached another record corresponding to 60 million tons, with an estimated total value of USD 119 000 million (FAO, 2012). However, wild fish capture (as anchovy, mackerel and blue whiting) to produce fish meal and fish oil, that constitute the main protein and lipid sources available within the animal feed, have been overexploited, decreasing from 10.7 million tons in 2004 to 4.2 million tons in 2010 (FAO, 2012). The farmed fish production requirements have exceeded the annual fish meal and fish oil production generating a sharp rise in prices of these products (Bostock et al., 2010) and also causing negative ecological impact for the environment and natural resources. This increased cost adversely affects the profitability of aquaculture industry, with aquafeeds accounting for 40-50% of production cost (Bórquez and Hernandez, 2009). This situation stimulated that plant protein sources begun to be tested in the incorporation of omnivorous fish (tilapia, catfish, carp, milkfish) and carnivores fish (salmon, trout, sear the tuna) diets (Hardy et al., 2010). The most common plant protein source incorporated to fish diet has been soybean flour. Diets for herbivorous and carnivorous fish normally contain between 15% and 45% of soybean meal. However, the uses of corn, peas, lupines, canola, barley, and wheat have been gradually increasing (Naylor et al., 2009). If the current level of fish production is maintained, it is anticipated that over the next 10 to 12 years, the aquaculture growth activity will depend strongly on plant protein sources (FAO, 2012). Up to date, researchers in farmed fish have investigated how new formulated diets affects fish growth. However, studies concerning to the identification of specific genetic variants among individuals that influence the growth phenotype in response to the diet have been limited.

In light of this scenario, arises the follow question: How do fish respond genetically (gene expression and genetic polymorphisms) and phenotypically (growth) when they are fed with a plant protein-based diet?

1.2. Nutritional genomics

This question can be addressed through studies of "nutritional genomics" which integrates nutrition and genetics on two approaches: Nutrigenomics and Nutrigenetics. Nutrigenomics focuses on studying how diet affects the modulation on gene expression patterns (Mutch et al., 2005). Nutrigenetics refers to how specific genetic variants influence the phenotype (growth) in response to the diet. (Mutch et al., 2005).

These genetic variants often occur as single nucleotide polymorphisms (SNP) in coding genes, which may influence the expression of a particular phenotype (Vignal et al. 2002; Liu and Cordes 2004). A SNP is a variation in DNA sequence that affects a single base (adenine (A), thymine (T), cytosine (C) or guanine (G)) of a genome sequence. They are the most abundant polymorphisms in genomes, have a codominant inheritance pattern and are adaptable to automated genotyping (Stickney et al. 2002; Liu and Cordes, 2004; Vignal et al., 2002).

1.3. Fish growth

Growth is a quantitative trait, controlled by polygenes with small effect on the phenotype and very often by major genes with larger effects as well as environmental factors (Falconer and Mackay, 1996).The changes in fish nutrition is one relevant factor that could affect multiple physiological functions, which are reflected in important production traits such as growth (Krogdahl et al., 2010). Plants protein have increased its importance as part in the incorporation of farmed fish diet, however plant proteins have different amino acid profile, metabolizable energy and minerals compared with fish meal protein that is usually an major ingredient of farmed fish (Kaushik and Seiliez, 2010). Plant proteins are deficient in some essential amino acids such as methionine, lysine and arginine, besides it has a variable digestibility, and is low in phosphorus. They also contain anti-nutritional factors (ANFs) such as protease inhibitors, lectins, antigenic proteins and phenolic compounds (Francis et al., 2001). However, ANFs can be reduced by different treatments such as heat and hulling before to be incorporated at fish diets (Kaushik and Seiliez, 2010; Krogdahl et al., 2010). Some studies in sea bream (*Sparus aurata)*, Atlantic salmon (*Salmo salar)* and rainbow trout (*Oncorhynchus mykiss*), reported a decrease in growth when these fish were fed with 50%, 75% and 100% replacement of fish meal with plant protein (Alami-Durante et al., 2010a; Gómez-Requeni et al., 2004; Mundheim et al., 2004). However, other studies in rainbow trout showed that fish meal may be replaced partially by soybean without affecting the growth of fish (Kaushik et al., 1995; Olli and Krogdahi, 1994).

1.4. Nutrigenomics studies in fish

Nutrigenomics studies have generated interesting insights about the effects of new formulated diets on gene expression in different tissues, as intestine and liver principally (Benedito-Palos et al., 2007; Froystad et al., 2008; Froystad-Saugen et al., 2009; Lilleeng et al., 2007; Panserat et al., 2008). However, effects in fish muscle have been scarcely documented; even when the muscle is an important tissue that represents about 60% of the total fish body weight (Roher et al., 2007; Alami-Durante et al., 2010a;

2010b). There is limited research that focuses on studying how plant protein-based diet affects the expression of candidate genes related to growth in relation to animal protein-based diet. Besides there is no information on the comparison in the expression of these kinds of genes among individuals of different growth rates, neither knowledge about genetic variants (SNP) associate with fish growth in response to new formulated diets. These aspects were carried out with nutrigenomics and nutrigenetics approaches in this thesis.

1.5. Candidate gene strategy

Candidate genes strategy consists on choosing genes according to the knowledge about their role in metabolic pathways and whose protein products are contributing with the phenotype under study. In this study eight genes involved in metabolic pathways or signal transduction that regulates growth fish (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Myostatin1b*) were selected to nutrigenomics approach. *Igf1a* is involved in the process of cell cycle, cell division, mitosis and protein transport (Moriyama et al., 2000); *Igf2a* controls the skeletal myogenesis and is involved in the AKT/mTOR pathway (the mammalian target of rapamycin), which contributes to the regulation of cell division (Clemmons, 2009); *mTOR* contributes to the regulation and protein synthesis involved in skeletal muscle hypertrophy (Kimball and Jefferson, 2006); *Pld1a* participates in cell growth regulation and the activation of *mTOR* through nutrient-sensing pathway (Yoon and Chen, 2008); myogenic regulatory factors (MRFs: *Mrf4*, *Myod1*, *Myogenin*) are involved in satellite cell activity (these are the cells that provide the new nuclei required for skeletal muscle during hypertrophic and hyperplasia) (Atchley et al., 1994; Hinits et al., 2007); and Myostatin1b (*Mstn1b*) is a key negative regulator of growth and muscle development (Acosta et al., 2005; McPherron et al., 1997). These genes also were used to examine the gene expression pattern in muscle of adult zebrafish with lower and higher growth in response to plant protein-based diet. In this study in order to select potential candidate genes to found genetic variation (SNP) that could be associated to growth phenotype, we identified all genes with different expression in muscle of adult zebrafish with lower and higher growth thought RNA-sequencing technology.

1.6. Advances in genomics tools: RNA-sequencing

RNA-sequencing is the first sequencing method that allows a quantitative analysis of the entire transcriptome at single base pair resolution (Morozova and Marra, 2008, Mortazavi et al., 2008, Wang et al., 2009). Besides, this technique can detect various sequence variation (SNP) in coding regions of all expressed genes in different tissues (Morozova and Marra, 2008, Mortazavi et al., 2008, Wang et al.,

2009). This technic could change the practice of candidate gene approach or microarrays experiments, generally used in nutrigenomics, moving toward a more functional understanding of the regulatory networks and pathways that underlying the quantitative phenotypic traits by finding genetic variation which could be associated to this phenotype(Cánovas et al., 2010, Wickramasinghe et al., 2011, Wickramasinghe et al., 2012).

1.7. Zebrafish as a model organism

In this study zebrafish was used as a model organism for nutritional genomics studies (Ulloa et al., 2011). The advantages to use zebrafish in this area are 1) their small generation interval allowing performing growth studies in a shorter time 2) The possibility to design mating as is used in fish farmed breeding program 3) studies can be conducted with a greater number of fish, ideal to perform a powerful data analysis to evaluate quantitative traits. 4) The laboratory rearing conditions are more homogeneous and reproducible than farmed rearing condition. 5) Finally, zebrafish are omnivores, they can eat a great variety of foods including animal and plant protein diets. On the other hand, there are a wide variety of molecular tools and information available for genomics analysis (Grunwald and Eisen 2002; Orban and Wu 2008). Studies using zebrafish provide opportunities for investigating the genetic mechanisms that underlies growth. Those results can subsequently be applied to species of commercial interest by comparative genomics.

1.8. Hypothesis

Considering that 1) fish growth results from the interaction between the genetic information of individuals and environmental factors, among which the diet is very important; 2) The diet components play a key role in regulating gene expression, 3) Besides, genetic variants (SNP) present in coding genes related with growth could contribute to phenotype differences.

For these reasons, the following hypotheses are presented:

1. Candidate genes that regulate fish growth (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Mstn1b*), are differentially expressed when zebrafish are fed plant protein-based diet in comparison to other fish fed fish meal protein-based diet.

2. Candidate genes that positively influence fish growth (*Igf1a, Igf2a, mTOR, Pld1a, Mrf4, Myod* and *Myogenin*) are over-expressed in zebrafish of higher growth as compared to lower growth. While *Mstn1b* that negatively influence growth is under-expressed in zebrafish of higher growth as compared to lower growth when fish are fed a plant protein-based diet.

3. The identifying of genetic variants (SNP) in differentially expressed genes are heterogeneously distributed in individuals of lower and higher growth in a zebrafish population fed with plant protein-based diet.

1.9. General objective

To examine differential gene expression related to growth in muscle of zebrafish in response to plant and animal protein-based diet and identify SNP in differentially expressed genes in adult zebrafish of lower and higher growth fed with plant protein diet to perform a growth association study.

1.10. Specific objectives

1. To examine the expression of candidate genes (*Igf1a, Igf2a, mTOR, Pld1a, Mrf4, Myod, Myogenin* and *Mstn1b*) in muscle of adult zebrafish fed with a plant protein-based diet and with fish meal protein-based diet.

2. To examine the expression of candidate genes (*Igf1a, Igf2a, mTOR, Pld1a, Mrf4, Myod, Myogenin* and *Mstn1b*) in adult zebrafish of lower and higher growth fed with a plant protein-based diet.

3. To identify allelic variants (SNP) in differentially expressed genes in muscle of adult zebrafish with lower and higher growth fed with a plant protein-based diet and perform a growth association study in a zebrafish population.

References

Acosta, J., Carpio, Y., Borroto, I., González, O., Estrada, M., 2005. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J. Biotechnol. 119, 324-331.

Alami-Durante, H., Médale, F., Cluzeaud, M. Kaushik, S., 2010a. Skeletal muscle growth dynamics and expression of related genes in white and red muscles of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture. 303: 50-58.

Alami-Durante, H., Médale, F., Cluzeaud, M., Kaushik, S., 2010b. Skeletal muscle cellularity and expression of myogenic regulatory factors and. Comp. Biochem. Physiol. 156:561-568.

Atchley, W., Fitch, W., Bronner-Fraser, M., 1994. Molecular evolution of the MyoD family of transcription factors. Proc. Natl. Acad. Sci. 91:11522-11526.

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J-A., Kaushik, S., Pérez-Sánchez, J., 2007. Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis. Aquaculture. 267:199-212.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L.,Handisyde, N., Gatward, I., Corner, R., 2010. Aquaculture: global status and trends. Phil. Trans. R. Soc. B. 365:2897-2912.

Bórquez, A., Hernández, A. 2009. Status and trends on the use of small pelagic fish species for reduction fisheries and for consumption in Chile In: Fish as feed inputs for aquaculture: practice, sustainability and implications. Hasan MR and Halwart M, (eds), pp. 289-324. FAO Fisheries and Aquaculture Technical Paper, Rome.

Canovas, A., Rincón, G., Islas-Trejo, A., Wickramasinghe, S., Medrano JF., 2010. SNP discovery in the bovine milk transcriptome using RNA-Seq technology. Mamm. Genome 21:592–598.

Clemmons, D., 2009. Role of IGF-I in skeletal muscle mass maintenance. Trend. Endocrinol. Metabol. 20:349-356.

Falconer D, MacKay T. 1996. Introduction to quantitative genetics. Longman Group Ltda., Malasia. Pp 464.

FAO (Food and Agricultural Organization). 2012. El estado mundial de la pesca y la acuicultura. Organización de las Naciones Unidas para la Alimentación y la Agricultura. Roma.

Francis, G., Makkar, H., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197-227.

FrØYstad, M., Lilleeng, E., Bakke-Mckellep, A., Vekterud, K., Hemre, G., Krogdahl, A. 2008. Gene expression in distal intestine of Atlantic salmon (*Salmo salar* L.) fed genetically modified soybean meal. Aquaculture Nutrition. 14:204-14.

FrØYstad-Saugen, M., Lilleeng, E., Bakke-McKellep, A., Vekterud, K., Valen, E., Hemre, G. 2009. Distal intestinal gene expression in Atlantic salmon (*Salmo salar* L.) fed genetically modified maize. Aquaculture Nutrition. 15:104-15.

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J., Médale, F., Martin, S., Houlihan, D., Kaushik, S., Pérez-Sánchez, J. 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232: 493-510.

Grunwald, D., Eisen, J. 2002. Headwaters of the zebrafish-emergence of a new model vertebrate. Nat. Rev. Genet. 3:717–724.

Hardy, RW. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Res. 41:770-6.

Hinits, Y., Osborn, DS., Carvajal, JJ., Rigby, PW., Hughes, SM. 2007. Mrf4 (myf6) is dynamically expressed in differentiated zebrafish skeletal muscle. Gene Expression Patterns. 7: 738-745.

Kaushik, SJ., Cravedi, JP., Lalles, JP., Sumpter, J., Fauconneau, B., Laroche, M. 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 133: 257-274.

Kaushik, SJ., Seiliez, I. 2010. Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquacult. Res. 41: 322-332.

Kaushik, S., Georga I., Koumoundouros, G. 2011. Growth and body composition of zebrafish (*Danio rerio*) larvae fed a compound feed from first feeding Onward: toward Implications on Nutrient Requirements. Zebrafish 8:87-95.

Kimball, SR., Jefferson, LS. 2006. New functions for amino acids: effects on gene transcription and translation. Am. J. Clin. Nutr. 83: 500S-507S.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S., Bakke, AM. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquacult. Res. 41, 333-344.

Liu, ZJ., Cordes, JF. 2004. DNA marker technologies and their applications in aquaculture genetics. Aquaculture 238:1-37.

Lilleeng, E., Froystad, MK., Ostby, GC., Valen, EC., Krogdahl, A. 2007. Effects of diets containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (*Salmo salar* L). Comp. Biochem. Physiol (Part A)147:25-36.

Mortazavi, A., Williams, B., McCue, K., Schaeffer, L., Wold, B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-seq. Nature Methods 5(7): 621-628.

Morozova, O., Marra MO. 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics 92: 255–264.

Moriyama, S., Ayson, FG., Kawauchi, H. 2000. Growth regulation by insulin-like growth factor-I in fish. Biosci. Biotechnol. Biochem. 64: 1553-1562.

Mundheim, H., Aksnes, A., Hope, B. 2004. Growth, feed efficiency and digestibility in salmon (*Salmo salar* L.) fed different dietary proportions of vegetable protein sources in combination with two fish meal qualities. Aquaculture 237: 315-331.

Mutch, MD., Wahli, W., Williamson, G. 2005. Nutrigenomics and nutrigenetics: the emerging faces of nutrition. FASEB 19: 1602-1615.

McPherron, AC., Lawler, AM., Lee, SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. Nature. 387, 83-90.

Naylor et al., Rosamond L. Naylor., Hardy RW., Bureau, DP., A, Chiu., Elliott, M., Farrell, AP., Forster, I., Gatlin, DM., Goldburg, RJ., Hua K., Nichols, PD. 2009. Feeding aquaculture in an era of finite resources. PNAS 106: 15103–15110.

Olli, JJ., Krogdahi, A. 1994. Nutritive Value of Four Soybean Products as Protein Sources in Diets for Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Reared in Fresh Water. Acta Agric. Scand. Sect. A. Animal Sci. 44: 185 - 192.

Orban., L, Wu Q. 2008. Cyprinids In: Genome Mapping and Genomic in Fishies and Acuatic Animal. Vol. 2. Ed. Springer. Germany. 195pp.

Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerré, D., Médale, F., Corraze, G., Kaushik, S. 2008. Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. Br. J. Nutr. 100:953-67.

Roher, J., Diaz, M., Mackenzie, S., Krasnov, A., Planas, J. 2007. Aproximaciones genómicas al crecimiento y nutricion de peces In: Monteros, J.E., (Eds.), Genética y Genómica en Acuicultura Madrid. pp 621-656.

Stickney, H., Schmutz, J., Woods, I., Holtzer, C., Dickson, M., Kelly, P., Myers, R., Talbot, W. 2002. Rapid Mapping of Zebrafish Mutations With SNPs and Oligonuleotide Microarrays. Genome Research 12:1929-1934.

Ulloa PE, Iturra P, Neira R, Araneda C. 2011. Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. Rev in Fish Biology and Fisheries 21:649–666.

Vignal, A., Milan, D., San Cristobal, M., Eggen, A. 2002. A review on SNP and other types of molecular markers and their use in animal genetics. Genet. Sel. Evol. 34:275-305.

Wang, Z., Gerstein, M., Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Genetics 10: 57-63.

Wickramasinghe, S., Hua, S., Rincon, G., Islas-Trejo, A., German, JB., Lebrilla, CB., Medrano, JF. 2011. Transcriptome Profiling of Bovine Milk Oligosaccharide Metabolism Genes Using RNA-Sequencing. PLosOne 6 (4): e18895.

Wickramasinghe, S., Rincon, G., Islas-Trejo, A., Medrano JF. 2012. Transcriptional profiling of bovine milk using RNA sequencing. BMC Genomics 13:45.

Yoon, MS., Chen, J., 2008. PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. J. Cell Sci. 121: 282-289.

Chapter 2. Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes.

Paper published in Review Fish Biology and Fisheries (2011) 21:649–666
DOI 10.1007/s11160-011-9203-0

Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes.

Pilar Ulloa[1,3], Patricia Iturra[2], Roberto Neira[3,4], Cristian Araneda[3]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile. Casilla 70061-7, Santiago, Chile

[3]Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile. Casilla 1004, Santiago, Chile

[4]AQUAINNOVO S.A., Polpaico 037, Puerto Montt, Chile.

Abstract

Zebrafish (*Danio rerio*) is a common research model in fish studies of toxicology, developmental biology, neurobiology and molecular genetics; it has been proposed as a possible model organism for nutrition and growth studies in fish. The advantages of working with zebrafish in these areas are their small size, short generation time (12–14 weeks) and their capacity to produce numerous eggs (100–200 eggs/clutch). Since a wide variety of molecular tools and information are available for genomic analysis, zebrafish has also been proposed as a model for nutritional genomic studies in fish. The detailed study of every species employed as a model organism is important because these species are used to generalize how several biological processes occur in related organisms, and contribute considerably toward improving our understanding of the mechanisms involved in nutrition and growth. The objective of this review is to show the relevant aspects of the nutrition and growth in zebrafish that support its utility as a model organism for nutritional genomics studies. We made a particular emphasis that gene expression and genetic variants in response to zebrafish nutrition will shed light on similar processes in aquacultured fish.

Keywords: Zebrafish, Growth, Nutrition, Nutritional genomics, Comparative genomics

2.1 Introduction

The zebrafish has become a very common research model in development, neurobiology and molecular genetics (Driever et al. 1994; Roush 1996; Bergeron et al. 2008). Zebrafish have recently been proposed as a possible model organism for nutrition and growth studies in fishes (Alestrom et al. 2006; Dahm and Geisler 2006; De-Santis and Jerry 2007; Wright et al. 2006; Johnston et al. 2008). Growth is a trait of main interest because it is closely linked with the productivity and profitability of aquaculture production (De-Santis and Jerry 2007). Phenotypic expression of this trait is under genetic control, but also depends upon environmental effects, and is directly affected by nutrition (Moriyama et al. 2000). Advances in genetic technologies have opened a window to understand the genetic variation underlying quantitative traits, among which the identification of candidate genes for growth and development is remarkable, along with the mapping of genomic regions of quantitative trait loci (QTL) which affect growth traits in cultured species (Davis and Hetzel 2000; FJalested et al. 2003; Reid et al. 2005; Araneda et al. 2008; Lo Presti et al. 2009; Dumas et al. 2010). In this aspect, the zebrafish has a wider variety of molecular tools and information available for genomic analysis than any other cultured species. The zebrafish has been proposed as a model fish for nutritional genomic studies, with the expectation that results from this approach will provide comparative genomic information applicable to aquacultured fish (Metscher and Ahlberg 1999; Drew et al. 2008; Robison et al. 2008; Crollius and Weissenbach 2008). Nutritional genomics is a discipline which integrates nutrition and genetics. Is a discipline that investigates gene-nutrient interactions using two approaches: “Nutrigenomics”, which studies how diet affects the expression of certain genes, and “Nutrigenetics”, which studies the genetic variants that influence the organism’s response to nutrients (Kaput et al. 2003; Müller and Kersten, 2003; Mutch et al. 2005; Marti et al. 2005; Panserat et al., 2007). In aquacultured fish some studies have been developed in nutrigenomics during the fasted-fed transition, as well as after the replacement of dietary fish meal or fish oil by plant ingredients (Panserat and Kaushik 2010). Investigating plant food sources became important due to the change of fish diets in the aquafeed sector, where plant derived ingredients are increasingly used due to the global demand of fishmeal (Hardy 2010; Turchini et al 2009). These nutrigenomic studies help us to understand how a change in the formulation of the diet produces multiple physiological responses in fish at the trasncriptional level.

Nutrigenetics refers to the analysis of specific genetic differences between individuals that respond in different ways to the same diet. These individual variations often occur as single nucleotide polymorphisms (SNP) in coding genes, which may influence the expression of a particular phenotype (Vignal et al. 2002; Liu and Cordes 2004). SNPs are point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. SNPs are becoming a focal

point in molecular marker development since they are the most abundant polymorphisms. In zebrafish there is one SNP for every 145-219 bp. They have a codominant inheritance pattern, are adaptable to automated genotyping, and reveal hidden polymorphisms not detected with other markers and methods (Stickney et al. 2002; Vignal et al. 2002; Liu and Cordes 2004). SNP variants may be responsible for the phenotypic variation of some important aquaculture quantitative traits such as growth related traits. Identification of functional polymorphisms in zebrafish is a step toward SNP association with productive traits (FJalested et al. 2003; Ryynänen et al. 2006; Lo Pestri et al. 2009; De-Santis and Jerry 2007). The main objective of this review is to present the most relevant aspects of the nutrition and growth in zebrafish that supports its utility as a model organism for nutritional genomics studies in aquacultured fish with special utility to salmonids.

2.2. Advantages of zebrafish as a model organism

Zebrafish have advantages biology as a model organism that include their small size, short generation time interval (12-14 weeks), their capacity to produce numerous offspring, breed easily and very amenable to manipulation in a laboratory tank (Clarck 2003). On the other hand, zebrafish have a wide variety of molecular tools and information available for genomic analysis, and a characterized genome compared to aquacultured fishes. The eighth revision (Zv8) of sequenced genome of the zebrafish was concluded in 2008 (Orban and Wu 2008) and recently in October 2010 the Zebrafish Genome Project with to the Genome Reference Consortium at NCBI (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/index.shtml) completed a new revision (Zv9). The existence of shared synteny between human and zebrafish genomes has been demonstrated (Barbazuk et al. 2000), as well as between zebrafish and green spotted pufferfish (*Tetraodon nigriviridis*) (Woods et al. 2005). Evidence obtained from rainbow trout (*Oncorhynchus mykiss*) genetic maps also show synteny between zebrafish and trout (Rexroad et al. 2005, 2008). Considering the synteny between genomes of different species and the existing evidence, recent revisions concluded that there should be abundant synteny between the zebrafish and salmonid genomes at the level of small regions that include conserved genes. In a sequencing study of ~1 Mb of Atlantic salmon, gene order in a region was conserved in three model fish species medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and zebrafish, but only in zebrafish was it possible to identify similar genes to salmon (Quinn et al. 2008). The generation of 43,000 BAC end sequences (BES) reported for catfish shows conserved syntenic regions between catfish and zebrafish genome. A total of 10,943 catfish BES (17,3%) had significant BLAST hits to the zebrafish genome, with 3,221 unique gene hits, providing a platform for comparative mapping based on locations of these catfish genes in the zebrafish genome (Liu et al. 2009). Thus, given the

synteny of these genomes, it is possible to use the genomic resources that zebrafish possess and adapt them for use in fish species of economic importance.

2.3. Genomic and genetic resources of zebrafish and other aquacultured fishes

The zebrafish genome has an average C-value of 1,848 pg (Animal Genome Size Database) and is estimated to contain $1.7x10^9$ base pairs of DNA which is about half the size of the mammalian genome (Postlethwait 2004). Zebrafish information available includes 35,204 genes; 1,481,937 ESTs (expressed sequence tags); 161,330 GSS (genome survey sequence) and 662,336 SNPs (National Center for Biotechnology Information; NCBI). In zebrafish 2,035 SNPs in 712 genes were identified by Stickney et al. (2002) and later Wood et al. (2005) developed a more complete map with 4,073 mostly SNP markers in genes and ESTs. Bradley et al. (2007) identified approximately 550,000 potential SNPs in 39% of the genomic sequence of zebrafish. They verified subgroups of these SNPs, found that over 70% may be validated polymorphisms and estimated that near 390,000 corresponded to available SNPs. Up to the present, SNP application in teleosts has been restricted to salmonids, using conserved sites (synteny) to validate SNPs between species (Smith et al. 2005).

On the other hand a rapid development of genetic and genomics resources has been made recently for economically important aquacultured fish species such as channel catfish (*Ictalurus punctatus*) (Li et al. 2007; Xu et al. 2007) rainbow trout (Rexroad III et al. 2003; Govoroun et al. 2006), Atlantic salmon (Thorsen et al. 2005; Davidson et al. 2010), Atlantic cod (*Gadus morhua*) (Nielsen et al. 2006; Wesmajervi et al. 2007; Johansen et al. 2009) and Gilthead sea bream (*Sparus aurata*) (Franch et al. 2006; Senger et al. 2006; Sarropoulou et al.2007).

Genomic information for Atlantic salmon includes 3,974 genes; 498,212 ESTs; 203,387 GSS and 1,344 SNP (NCBI, January 2011). Recently 9,057 full-length reference genes were characterized in this salmon (Leong et al. 2010), and several SNP markers have been discovered. For example, 2,507 putative SNPs from the alignment of 100,866 ESTs were discovered by Hayes et al. (2007). In the same way Boulding et al. (2008) identified 129 SNPs within ESTs spaced throughout the linkage map and identified 79 SNPs markers on linkage groups associated with QTLs for growth rate, condition factor, shape and skin pigmentation traits. Moen et al. (2008a) developed 1,369 SNPs markers of which 304 are located within genes anchored in a linkage map. A panel of 15,225 SNPs from the Atlantic salmon SNP chip (Center for Integrative Genetics, CIGENE) was evaluated as a potential genomic tool for whole genome selection (WGS), where 2,991 single copy polymorphic SNPs were considered useful for further analysis Domink et al. (2010).

Genetic resources for catfish include over 500,000 ESTs (Wang et al. 2010; Li et al. 2007), over 10,000 full-length cDNA (Chen et al. 2010), over 300,000 putative SNPs (Wang et al. 2010), and the development of high-density SNP chip is in progress (Lu et al. 2010). In Atlantic Cod 724 putative SNP developed from 17,056 EST were evaluated, from which 318 segregating SNPs were selected for future genetic analysis (Moen et al. 2008b). In sea bream a long-read EST database was generated, containing 1,393 cDNA clones representing 852 unique cDNA sequence-reads (Sarropoulou et al. 2005). Seventy-six SNPs markers for use in studies of natural populations and selective breeding were validated in this species (Cenadelli et al. 2007). The large numbers of SNP discoveries in different species represent a valuable genetic resource and may be used for performing genome scans of genes or of quantitative trait loci (QTL) influencing commercially important traits by comparative genomic analysis.

The most powerful genomic resource in zebrafish is the accessibility of its complete genome sequence (Grunwald and Eisen 2002; Orban and Wu 2008). An International Collaboration by Canada, Chile and Norway is being undertaken to obtain a similary complete sequence of the genome of Atlantic salmon (Davison et al. 2010). Also, efforts to sequence the complete genome of Atlantic cod and catfish has been initiated and is currently in progress (Johansen et al. 2009; Lu et al. 2010). Given that these genome sequencing projects from different aquacultured fishes are underway, the research community can find available information from diverse data sources (Table 2.1). Fish Map is a unified and centralized resource for storage, retrieval, and display of genomic information on zebrafish. FishMap is built on the Gbrowse, and is a part of the Generic Model Organism Database Consortium Project (GMOD) (Meli et al. 2008). cBARBEL (Catfish Breeder and Researched Bioinformatics Entry Location) is an important catfish genome database and represents one of the first comprehensive bioinformatic databases for an aquaculture species (Lu et al. 2010). Salmon DB a new Chilean multi-organism public access database (on registration) has been recently created. It contains EST sequences from Atlantic salmon, rainbow trout and whole genome sequences from five model fish species (zebrafish, medaka, pufferfish, fugu and stickleback). It was built using the core components from the Generic Model Organism Database (GMOD) project and the Gene Ontology and Pathways Architecture (GOPArc) system (Table 2.1).

Table 2.1. The main web-based data sites currently available for zebrafish and aquacultured fishes (according to Briggs 2002; Clark 2003; Meli et al. 2008; Johansen et al. 2009; Lu et al. 2010)

Specie	Website Name	Internet Link
Zebrafish (*Danio rerio*)	Zfin	http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg
	NCBI Zebrafish Genome Resources	http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/index.html
	Entrez Map Viewer	http://www.ncbi.nlm.nih.gov/projects/mapview/static/MapViewerHelp.html
	Ensembl genome browser	http://www.ensembl.org/Danio_rerio/Info/Index
	Zebrafish Mutation Resource	http://www.sanger.ac.uk/Projects/D_rerio/mutres/
	DFCI Zebrafish Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=zfish
	FishMap	http://fishmap.igib.res.in
Rainbow trout (*Oncorhynchus mykiss*)	Rainbow Trout Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=r_trout
	NAGRP Aquaculture Genome Projects	http://www.animalgenome.org/cgi-bin/host/rainbow/viewmap
	Sigenae Contig Browser	http://public-contigbrowser.sigenae.org:9090/Oncorhynchus_mykiss/index.html
	SalmoDB	http://genomicasalmones.dim.uchile.cl/
Atlantic Salmon (*Salmo salar*)	Atlantic Salmon Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=salmon
	cGRASP consortium	http://web.uvic.ca/cbr/grasp
	SalmoDB	http://genomicasalmones.dim.uchile.cl/
	Asalbase	http://www.asalbase.org/sal-bin/index
Catfish (*Ictalurus* spp.)	cBARBEL	http://catfishgenome.org
Atlantic Cod (*Gadus morhua*)	Atlantic Cod Genomics and Broodstock Development	http://www.codgene.ca
	The Cod Genome Project.	http://www.codgenome.no

2.4. Relationships of zebrafish and other fish models

Five teleosteos fish genomes have been fully sequenced including zebrafish (from Cypriniformes), Japanese fugu (*Fugu rubripens*, from Tetraodontiformes), green spotted pufferfish (*Tetraodon nigroviridis*, from Tetraodontiformes), medaka (from Beloniformes), and three-spined stickleback (*Gasterosteus aculeatus*, from Gasterosteiformes). Fugu and pufferfish have been sequenced by whole genome shotgun (WGS) assembly (Aparicio et al. 2002). They have very small genome sizes, low nuclear DNA content, less than 500 million base pairs (Mb) per haploid genome (Aparicio et al. 2002; Clark 2003), and have been selected as a model for the human genome project (Cossins and Crawford 2005). However, both fugu and tetraodon will only be used as model genomes, but not as model organism because they do not breed easily in captivity (Westerfield 2000; Lawrence 2007; Clark 2003). Stickleback is a model fish used to addres questions related to adaptation, speciation, and evolution (Bell 2001; Cresko et al. 2007). It is a small fish (adult total body length < 10 cm), survives and breeds in both seawater and freshwater; displays a variety of pronounced reproductive behaviors; has a simple and short life cycle of one year in nature, but can be brought to sexual maturity in the laboratory within 7 -8 months after hatching (Hahlbeck et al. 2004; Katsiadaki et al., 2002).

Medaka is another model fish with a genome size estimated at 650-1,000Mb, its genome is one-third the size of the human genome and is less than half the size of the zebrafish genome (Hinegardner et al. 1972; Kasahara et al. 2007, Clark 2003). Medaka, tolerate a wide range of salinities and temperatures (10-40°C), are small (3-4 cm), have eggs transparent, are easy to breed and with a generation interval time of 6 to 8 weeks (Wittbrodt et al. 2002). Medaka and zebrafish phylogenetically are separated from their last common ancestor ~ 110 million years (Myr) ago (Figure 1). This evolutionary distance is reflected in many aspect of their biology, including their early development. With an evolutionary distance of ~60 Myr (compared with 110–160 Myr in zebrafish), medaka is a much closer relative to fugu and stickleback than it is to zebrafish (Figure 2.1), while zebrafish represent a more ancestral condition.

Zebrafish have a much to offer as a model fish in nutrition genomic studies, but they certainly are not perfect. In most vertebrates growth is determined, which means that growth ends at a given moment in the life of the animal and they reaches a finite size. In contrast, fish muscle growth is not lineal and occurs through a combination of recruitment of new muscle fibers (hyperplasia) and an increase of the size of existents fibers (hypertrophy) in post-juvenile stages (Johnston 1999; Mommsen 2001). In contrast to other fishes, zebrafish have determinate growth, the number of muscle fibers is established and fixed at

the time of the birth; and growth is solely due to hypertrophy of fibers already formed (Du 2004). This condition does not provide a general model for muscle growth since it only reaches a modest ultimate body size (3-5cm) (Johnston 1999; Mommsen 2001). However muscle differentiation, somite formation and subsequent differentiation of myoblasts are under the control of complex signaling pathways and of myogenic regulatory factors that are similar in all teleosteos (Du 2004). Understanding how these signaling cascades coordinate with others and how they control myogenic transcriptional networks which change gene expression during muscle differentiation may be unraveled through nutrigenomic studies in zebrafish.

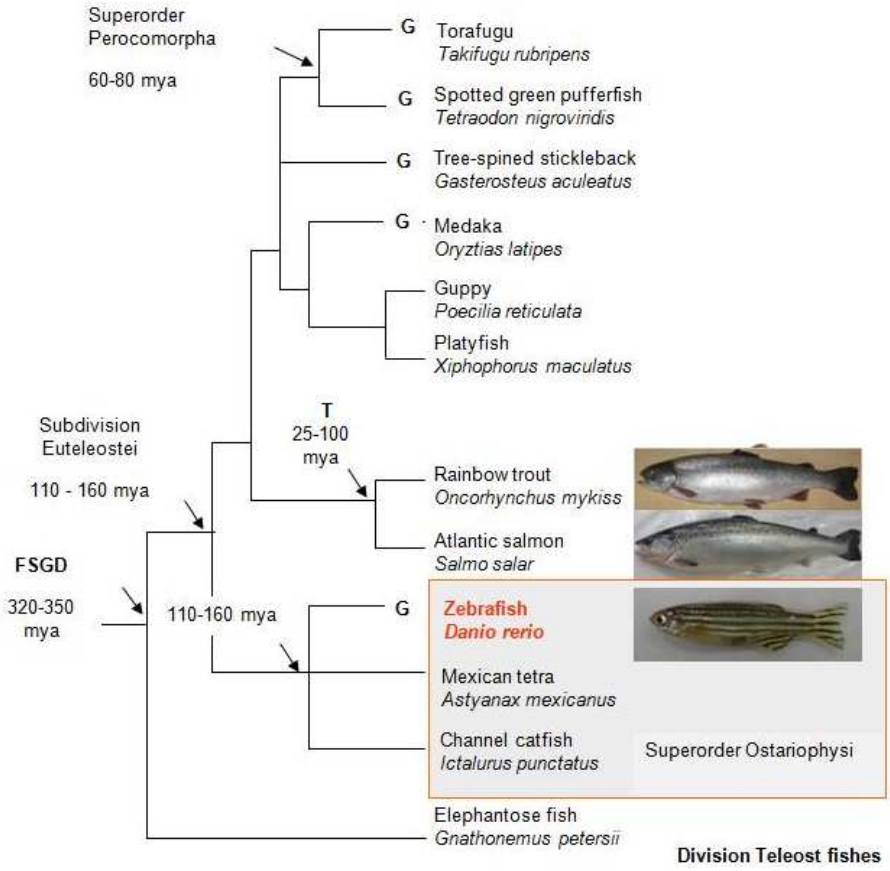

Figure 2.1. Teleost relationships of the most commonly studied teleosts. This genealogical tree illustrates fish-specific genome duplication (FSGD), salmonid-specific tetraploidization (T) and fish genome assembly (G). Notably, zebrafish represents a more ancestral condition compared to other model organisms as such medaka. These conditions enabling some translational biological processes addressed more effectively in zebrafish (Adapted from Wittbrodt et al. 2002; Volff 2005).

2.5. Phylogenetic relationships of zebrafish and aquaculture fish species

The last common ancestor of all the vertebrate laboratory models was the common ancestor of the Actinopterygii (ray-finned fishes) and Sarcopterygii (lobe-finned fishes and tetrapods), which probably lived during the Silurian period, approximately 420 million years ago (Janvier 1996; Hedges 2002; Clark 2003). Within the ray-finned fishes the dominant group today is the Teleosts which has over 20,000 species, including important aquaculture species such as the Atlantic salmon, rainbow trout, sea bass , and carp (*Cyprinus carpio*) (Clark 2003). The teleosts began a major evolutionary radiation in the Triassic, about 200 million years ago, and have since undergone massive diversification in morphology, physiology and habitat. Their genomes did not remain static and they are still evolving. Their evolutionary divergence and extreme diversity provide an abundant source of different genomes with all the structure-function combinations that have survived during the last 400 million years (Volff 2005). The class Actinopterygii includes those taxa considered as the most ancient fishes (Lauder and Liem 1989). As a group, they are characterized by many derived characteristics which are absent in primitive ray-fins. Teleosts are thus morphologically remote from the common actinopterygian/sarcopterygian ancestor. However, within the Teleosts, the Ostariophysi (and thus zebrafish) retain many primitive characteristic and occupy a relatively basal position (Lauder and Liem 1989). Thus the zebrafish is a rather generalized teleost and can, in most cases, be used to represent the “primitive” or “ancestral” condition in comparison with more modern laboratory teleosts such as the medaka and fugu (Lauder and Liem 1989; Metscher and Ahlberg 1999) (Figure 2.1).With an evolutionary separation of less than 150 million years, the zebrafish is still closer to the aquacultural fish species than any mammalian model organism such as a mouse , whose common ancestor with the teleosts lived around 400 million years ago (Dahm and Geisler 2006). This evolutionary proximity between zebrafish and aquacultured fish species is reflected in many aspects of their biological similarity, and offers several advantages combining the power of genetics with experimental embryology, molecular biology and genomic resources (Wittbrodt et al. 2002).

The plasticity of fish genomes could be manifested by the size of their genome and chromosome number. The haploid zebrafish genome has 25 chromosomes which is similar of the karyotype of the original teleost (Ojima 1983; Daga et al. 1996; Gornung et al. 1997) after an event of fish genomic duplication (n =24; Woods et al. 2005). Most teleosts have around 24-25 haploid chromosomes with very little variation, except in the case of certain lineages such as the salmonids, which contain about 30-40 haploid chromosomes (Allendorf and Thorgaard 1984; Clark 2003; Naruse et al. 2004). Chromosome number distribution in teleosts is quite restricted, in contrast to the broadly distributed chromosome number observed in tetrapodes (Naruse et al. 2004). Comparative genome analysis of medaka and zebrafish with

reference to the human gene map indicates that karyotype evolution in teleosts occurred by chromosome fragmentation; mainly inversions (intra-chromosomal rearrangements) and by translocations (inter-chromosomal rearrangements), but not by fusion and/or fission of chromosomes as is observed in mammalian chromosome evolution (Ehrlich et al. 1997; Naruse et al. 2004). Taken together these findings, suggest that teleost karyotypes and the gene content of individual chromosomes should be more similar to the karyotype of the last common ancestor of ray-fin and lobe-fin fish than that of most mammalian genomes (Postlethwait 2004; Woods et al. 2005).

2.6. Life cycle of the Zebrafish

In typical laboratory conditions (28.5° C and 14:10 light-dark hours), zebrafish develop very rapidly. Embryogenesis takes 24 hours post fertilization (hpf) (Dahm 2002). Larvae hatch within 2.5-3 days post-fertilization (dpf) and the transition from larva to juvenile (metamorphosis) occurs at ~30 dpf (Kimmel et al. 1995). Zebrafish reach sexual maturity from 10 to 14 weeks post-fertilization (Eaton and Farley 1974a). Under favorable conditions zebrafish spawn continuously after sexual maturation (Eaton and Farley 1974b; Breder and Rosen 1966). Their high reproductive capacity and their short generation time (12-14 weeks) serve as an advantage over cultured fishes that present a long generation interval (for example in salmonids this generation interval is 2 to 4 years, depending upon the species) (Wegrzyn and Ortubay 2009). In a typical domestic salmonid such as the anadromous rainbow trout, once fertilization occurs the incubation period depends essentially on temperature, requiring about 30 days at 10° C or 300 accumulated thermal units (ATU) to reach 50% hatching (Estay et al. 1994). Juvenile fish or “parr”, identifiable by their vertical and black spots on both sides of the body (with 1,200 ATU; 3 – 15g weight), remain one year in freshwater until smoltification (a metabolic processes which adapts a fish from freshwater to marine water); smolt (with 3,200 ATU; 80 – 120g) spend another year in marine water until harvest (3 - 4.5 Kg). Depending on the quality of food and genetic factors, sexual maturity is reached in males in the second or third year of life and in females in the third or fourth year (Wegrzyn and Ortubay 2009). These conditions: long generation interval and late sexual maturity, limit population level studies. A comparative view of zebrafish and rainbow trout life cycles, giving the time periods for their different stages of life, is shown in Figure 2.2.

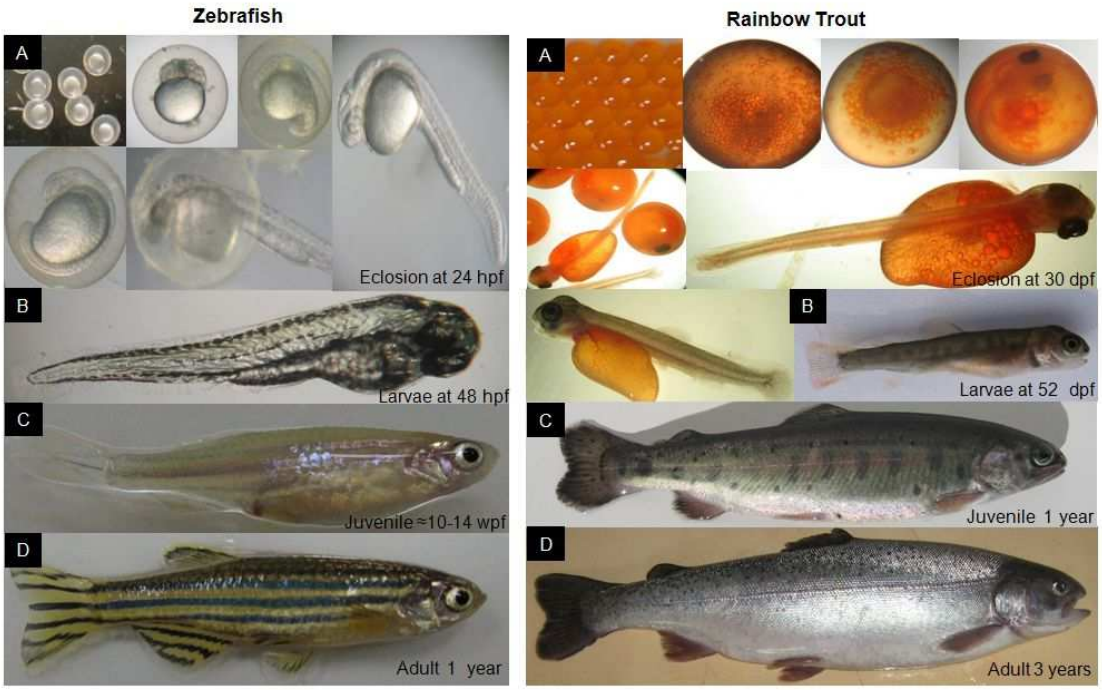

Figure 2.2. Comparative view of the life-cycle of zebrafish and rainbow trout a Zebrafish eggs (1 mm of length), the different stages of embryo development at 28^{o}C and eclosion at 24 hpf; Rainbow trout eggs (5 mm of length) the different stages of embryo development at 10^{o}C, and eclosion at 30 dpf or 300 ATU. b Zebrafish larvae at 48 hpf (size ~ 2 mm length); Rainbow trout larvae at 52 dpf or 520 ATU (size ~ 2.5 cm length) c Juvenile zebrafish reach sexual maturity between 10–14 weeks post fertilization (size ~21 mm length); Juvenile rainbow trout identifiable by their parr spots (size ~20 cm length) d Adult zebrafish with common phenotype at ~ 1 year old (size 3–5 cm length); Adult rainbow trout reach sexual maturity at 2–3 years old (size ~ 40 cm length). The images of embryonic development (zebrafish and trout) were obtained with a Canon PowerShot SD550 digital camera and a stereoscopic zoom microscope Nikon SMZ-10 (20–1609 in optical magnification).

2.7. Digestive system of adult zebrafish

Fish have different feeding structures, mechanisms and habits. They may be classified broadly by their feeding habits as detritivores, herbivores, omnivores, or carnivores (Moyle and Cech 2000; Horn et al. 2006). Within each category, organisms may be thought of as either euryphagous (eating a great variety of foods), stenophagous (eating a limited variety of foods), or monophagous (eating only one type of food) (Moyle and Cech 2000). The majority of aquacultured fish are either euryphagous carnivores (such as salmon, bass, bream, halibut, turbot, flounders, and groupers), euryphagous omnivore (such as channel catfish, tilapia and zebrafish), or euryphagous herbivore (such as carp). The zebrafish has a long intestine with a large absorption area and no true stomach (Figure 2.3), while carnivore fish species (salmon and trout) have a short intestine and a true stomach (Moyle and Cech 2000; revised in Rust 2002). Studies have shown that carnivorous fish, such as salmon, have a compensatory mechanism that allows them to

modify and adapt their digestive and physiological systems in response, to a vegetal diet. Rainbow trout and sea bream, show gut augmentation when they are fed vegetal protein diets (over 75% fish meal replacement) (Santigosa et al. 2008). The vegetable diet decreases digestive activity, increaseds gut length and enzymatic activity of enzymes such as trypsin, and produceds a growth rate similar to fish fed with fish meal (Santigosa et al. 2008). Other evidence shows that gut length in fish is affected by ontogeny, diet and phylogeny. For example, gut length was measured in four fish species, two herbivores (*Cebidichtys violaceus* and *Xiphister mucosus*) and two carnivores (*X. atropurpureus* and *Anoplarchus purpurescens*), all fed with a high-protein artificial diet (≥ 55% protein from marine animal sources). In herbivore species there was no ontogenetic change in gut dimensions on the high-protein artificial diet, suggesting that herbivores are genetically programmed to develop relatively large guts. However, *X. atropurpureus* (carnivore) increased its gut dimensions reaching a size similar to its sister taxon, *X. mucosus* (herbivore), suggesting a phylogenetic influence, but it also decreased its gut dimensions on the high-protein artificial diet, suggesting phenotypic plasticity (German and Horn 2006). These results show that the anatomical pattern of herbivore and carnivore vertebrates does not appear to be conserved in fishes, and show a phenotypic plasticity (ontogenetic increase) in the length of the digestive tract even in carnivorous species. These observations allow us to suppose that differences between the zebrafish digestive system and that of salmonids would not be a constraint when using the zebrafish as a model in nutritional studies.

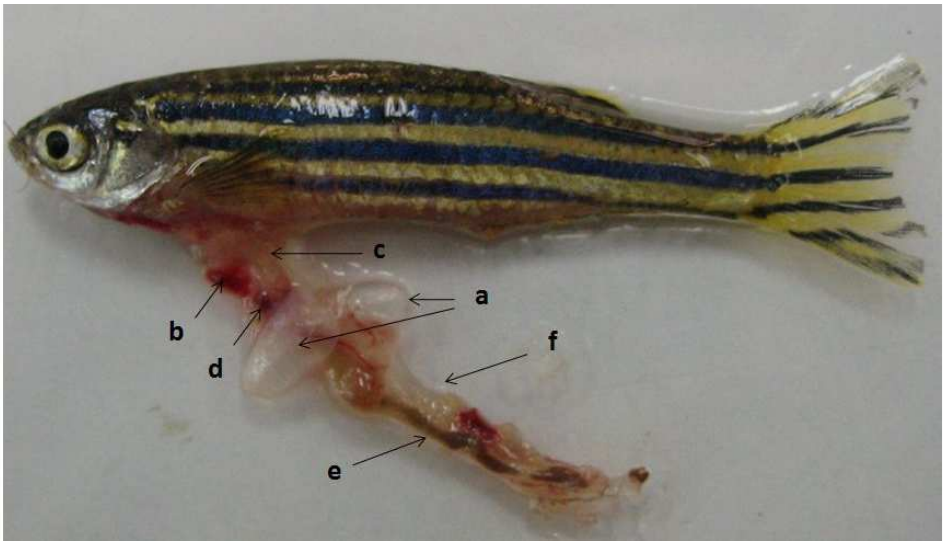

Figure 2.3. Digestive tract from *Danio rerio*. (a) Swim bladder, (b) liver, (c) esophagus, (d) spleen, (e) intestine, and (f) pancreatic tissue (around the gut). The image was obtained with a Canon PowerShot SD550 digital camera under normal conditions employing the internal zoom (39 optical magnification).

2.8. Diet of the zebrafish

Information on the dietary preferences of wild zebrafish is limited. Apparently wild zebrafish are generalists, consuming a wide variety of benthic and planktonic crustaceans, worms and insect larvae (Dutta 1993). McClure et al. (2006) and Spence et al. (2007) analyzed gut contents of sampled zebrafish in natural habits and found that insects, mostly of terrestrial origin, were the predominant prey; the diet also included a small percentage of plankton**,** whose main components identified were zooplankton and insects (58.6%), invertebrate eggs and arachnids (2.5%), phytoplankton (5.9%) and other unidentified components (33%). Zebrafish in captivity may be fed with live prey such as brine shrimp (*Artemia sp.*), rotifers (*Brachionus sp.*), artificial diet, or some mixture of both (Watanabe et al. 1983). These results show that zebrafish can feed on animal and on vegetal protein sources, and suggests that they may have similar digestive mechanisms to both of herbivores such as carp and tilapia, and carnivores such as trout and salmon. It also suggests that they would be able to modify and adapt their digestive system in response to diet, which could facilitate the design of diets and feeding protocols that better reflect the specific digestive physiology and feeding behavior of the zebrafish.

2.9. Nutritional requirements of zebrafish

Fish nutritional requirements must be determined for each life stage: larva, juvenile, and adult, through controlled experiments that test the effects of different dietary components on survival, growth, disease/stress resistance and reproduction. Nutritional requirements are unknown for zebrafish, even though it is a widely used as a model organism in laboratories (Lawrance 2007). In the guide for laboratory use of zebrafish, specific nutritional data are not discussed (Westerfield 2000). This is due in part to the fact that zebrafish can be maintained and successfully spawned under a wide variety of diets and feeding regimes, including those that are likely sub-optimal (Lawrence 2007).

Lipids, particularly fatty acids (FA), are well known dietary requirements that supply energy and act as pheromones, hormones, and membrane components. Fish are unable to synthesize *de novo* polyunsaturated fatty acids (PUFA), including the essential ones such as linoleic (18:2 ω-6) and linolenic acid (18:3 ω-3). Therefore, fish must obtain these substances from their food. Cold water fish have a higher requirement for ω-3 FA than ω-6 FA, whereas warm water species require both ω-3 and ω-6 FA (Meinelt et al. 2000). Zebrafish, like many other warm-water fishes, require higher amounts of ω-6 fatty acids (Watanabe 1981; Cowey and Sargent 1976). Meinelt et al. (1999, 2000) raised zebrafish on formulated feed containing varying ratios of ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), and found that growth and fertilization rates were positively correlated with the level of ω-6 PUFA in diet.

Protein requirements of juvenile omnivore, carnivore and herbivore ornamental fish are in accordance with the requirements reported for fish feeding (National Research Council 1993). The best approximation to assess zebrafish diet any requirements can probably be drawn from studies of cyprinid species such as the common carp and goldfish (*Carasius auratus*) (Lochman and Phillips 1996; Carvalho et al 2004; Dabrowski et al. 2005). Protein requirements vary from 30% to 53% in diets for optimal growth in goldfish (Fiogbé and Kestemont 1995). Westerfield (2000) proposed the feeding of adult zebrafish with a commercial diet of flake food for tropical fish; and for feeding larvae, the use of fine powder commercial food specific for these commercial foods have from 46% to 50% protein content. In relation to carbohydrates, Robison et al. (2008) examined the effect of manipulating dietary carbohydrate levels (0%, 15%, 25% and 35%) from the larval stage until sexual maturity in zebrafish. They demonstrated that long term manipulation of dietary carbohydrate levels had a significant effect on body weight gain, condition factor and body composition. More research on nutrient requirements of zebrafish is needed to gain comprehensive knowledge about to determine optimal dietary levels.

2.10. Growth rate in fishes

Growth is a quantitative trait and it may be measured at different times of the zebrafish life cycle. Many variations occur in zebrafish growth rates in laboratories (Eaton and Farley 1974b). This phenomenon is known as growth compensation or size-hierarchy effect (Brown 1957; Ricker 1958). It is particularly troublesome when using fish for experiments, where uniform growth and high survivability are important. Quantitative genetic variability of reproduction and growth performance using four zebrafish populations of different origins from Asia and Europe were studied. Traits of body length and the weight of progeny were measured every two weeks until 20 weeks of age. The average weight and length for the populations at 4, 12 and 20 weeks were 3.5; 74.9; 213.4 mg and 6.4; 15.3; 21.3 mm, respectively, with a high phenotypic correlation between the traits (r =0.95) (Von Hertell et al. 1990). Goolish et al. (1999) assessed the effectiveness of 10 artificial diets**.** A good performance was achieved with a commercial diet, leading to a maximum larval survival of about 60% and a standard length of 6.4 mm at 21 dpf . Later Biga and Goetz (2006) reported an average total length of 4.7 mm at 28 dpf when only paramecia were used to feed larvae. Carvalho et al., (2006) demonstrated that zebrafish larvae could be reared without live food with an admissible growth and a high survival (7.9 mm and 84% respectively), on the condition that food was presented in a continuous manner. When feeding larvae with artemia, the value obtained for standard length was 14.3 mm at 21 dpf, with a high larval survival of 86%. Santos et al. (2006) successfully used a mix of purified diet and artemia to feed zebrafish larvae, obtaining more than 70% survival at 120 dpf. These differences are indicative of discrepancies in rearing densities and in specifically diet, where growth is directly affected by nutrition and the feeding environment.

A quantitative trait such as growth is normally under control of polygenes, where each has a small effect on the phenotype, as well as environmental influence and a possible influence of major genes and/or QTLs (Falconer and Mackay 1996). Little research has been done to identify growth QTLs in aquaculture fish (Araneda et al. 2008). However, in zebrafish QTLs for growth rate and muscle fat content have been identified on chromosome 23 (Wright et al. 2006). This result is valuable because it is the first step in to identifing loci affecting these traits. Heritability estimations of growth rate in the main aquacultured fish species (salmon, trout, tilapia and carp) fluctuate from 20% to 41% (Tave 1993). Heritability estimated for weight and length at 4 and 20 weeks old in zebrafish were measured, were found to have very high values (0.17 and 0.40, respectively) (Von Hertell et al. 1990). This result agrees with estimates obtained in our laboratory for heritability of growth rate (wet weight) in 14 week-old zebrafish (0.42 ± 0.10) (Accini 2009).

2.11. Genes associated with growth in fishes

Growth traits have been under selection in fish and livestock breeding programs. Many genes associated with muscular growth (somatogenesis) have been mapped and sequenced, and have been shown to be conserved among mammals and fish (Lo Pestri et al. 2009). These results allowed the identification of numerous candidate genes involved in muscular growth of different fish species (De-Santis and Jerry 2007; Johnston et al. 2008). Among the many possible growth-regulating pathways in vertebrates, genes within the somatotropic axis and transforming growth factor superfamily have been the most targeted candidate genes in fish (De-Santis and Jerry 2007). The somatotropic axis essentially consists of growth hormone-releasing hormone (GHRH), growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factors (IGF-I and II), associated carrier proteins (IGFBPs) and receptors. These genes represent the main endocrine and autocrine regulators for skeletal muscle growth and are known to play a key role in the regulation of metabolism and of physiological processes (Moriyama et al. 2000; De-Santis and Jerry 2007; Johnston et al. 2008). The autocrine *IGF-II* transcription factor required for skeletal myocyte differentiation is regulated by *mTOR*, as well as by amino acid sufficiency. The mTOR-IGF axis is a molecular link between nutritional levels and skeletal muscle development (Erbay et al. 2003). In rainbow trout the evidence suggests that insulin and amino acid regulate TOR signaling (Seiliez et al. 2008). Another somatotropic axis hormone is leptin, which is thought to have a minor role in GH regulation. The presence of the *leptin* gene in teleosts initially was uncertain (Johnson et al. 2000), but now this peptide is known to be expressed in fishes and its DNA sequence has been found in the pufferfish and in the common carp (Kurokawa et al. 2005b; Huising et al. 2006). Structural growth in teleosts is mediated by growth factors (GFs) and myogenic regulatory factors (MRFs), generally produced in the muscle tissue (Tan et al. 2002).

Myostatin (MSTN), also known as growth/differentiation factor-8 (GDF-8), is a molecule belonging to the transforming growth factor β (TGF-β), which acts as a negative regulator of muscle development (McPherron et al. 1997). In fish, the *Mstn* mRNA has been detected in several tissues including gill, renal and gonadal tissues. These findings indicate that *Mstn* is involved in numerous physiological activities, and has been characterized in many fish species (rainbow trout, Atlantic salmon, sea bream, tilapia, catfish, among others) (De-Santis and Jerry 2007). *Mstn* is conserved as two genes in most teleosts (*Mstn 1 y Mstn 2*), as a result of the basal wide genome duplication (Maccatrozzo et al. 2001). In adult zebrafish subjected to environmental stress, both *Mstn-1* and *Mstn-2* genes expression were expressed in a wide variety of tissues (Helterline 2006). Acosta et al. (2005) reported that the inactivation of the *Mstn* gene in zebrafish resulted in an increase of weight gain (45% more compared to the control), as was also observed in mice by McPherron et al. (1997). Previous studies in mammals have demonstrated that myostatin is binds to the two activin type II receptors (*ActRII*) (Lee and McPherron 200; Rebbapragada et al. 2003). In zebrafish, *ActRII* and *ActRIIB* were shown to exhibit distinct roles in craniofacial development (Albertson et al. 2005). In Atlantic salmon, full-length ActRIIB cDNA was isolated and characterized by *in situ* hybridization and by RT-PCR in skeletal muscle (Ostbye et al. 2007). Carpio et al. (2009) examined the effect of administrating a recombinant soluble form of goldfish ActRIIB extracellular domain (G-ActRIIBed) to juvenile and larval goldfish, African catfish larvae (*Clarias gariepinus*) and tilapia larvae, it was found that this recombinant molecule stimulated growth in teleost fish in a dose-dependent manner.

Myogenic regulatory factors (MRFs) (*myogenin*, *myod*, *myf-5*, *myf-4* or *myf-6*) constitute a small family of transcriptional factors, called the *myod* gene complex, involved in myogenesis and are expressed exclusively in the skeletal muscle of vertebrates (Atchley et al. 1994). Genes belonging to the *myod* complex have been characterized only in a few fish species such as in the common carp and in rainbow trout (Kobiyama et al. 1998; Johansen and Overturf 2005). In zebrafish, *myf-5* and *mrf-4* have been also identified (Chen et al. 2001; Hinits et al. 2007; Wang et al. 2008).

Another gene thought to be essential to growth in fish, is follistatin (*fst*). It is a secreted glycoprotein that was first identified as a potent inhibitor of follicle-stimulating hormone secretion which was explained by its strong binding affinity to the TGF-β protein activin (Phillips and Krestor 1998). Follistatin inhibits the transformation of growth factor-β proteins and is a known regulator of amniote myogenesis (Macqueen and Johnston 2008). Phylogenetic analyses suggest that one *fst* gene (*fst1*) is common to euteleosts, but a second gene (*fst2*) is conserved specifically within the Ostariophysi. Zebrafish *fst1* and *fst2* map respectively on chromosomes 5 and 10 in two genomic regions, and share a each conserved synteny to a region in tetrapods (Macqueen and Johnston 2008). Other growth genes studied in fish are those of the calpain-calpastatin (*CAST*) system. This system is important because it regulates a wide range of

physiological processes including protein turnover and growth, cell cycle progression, and myoblast differentiation (Goll et al. 2003). Full-length cDNA has been obtained for *calpain 1* and *2* from rainbow trout, and show 65% identity to mouse orthologues (Salem et al. 2005a). Also, *calpain 1*, *calpain 2* y *calpastatin* gene expression was measured in rainbow trout subjected to starvation for 35 days. Rainbow trout starvation resulted in the up-regulation of mRNA transcripts for these three genes (Salem et al. 2005a). Salem et al. (2005b) report in a second study, two *CAST* isoforms for rainbow trout: a long (*CAST-L*) and a short one (*CAST-S*). Lastly, genes from the ubiquitin-proteosome proteolytic pathway are considered to be major in route of protein degradation involved in skeletal muscle and are regulated by nutritional status. The identification of all of these genes contributes to the understanding of the mechanisms that regulate growth in non-mammalian vertebrates and suggests a powerful biotechnological approach to improve aquacultured fish growth; however, the expression of nutritional regulation of these growth trait genes is poorly documented in fishes.

2.12. Zebrafish nutritional genomics

Nutrigenomic studies are being directly developed in the most important aquacultured fish species using microarrays and RT-PCR analysis, which allows the observation of differential gene expression in response to diets containing proteins from different vegetal origins, and in response to diets of different oil compositions (Froystad et al. 2008; Froystad et al. 2009; Lilleeng et al. 2007; Panserat et al. 2008; Douglas 2006). These nutrigenomic studies have generated basic knowledge about unchanged, over-expressed and under-expressed genes in response to different diets. Some nutrigenomic studies have observed the particular expression of some candidate genes correlated with growth in aquacultured fishes such as rainbow trout and Atlantic salmon under starvation and feeding conditions (Chauvigné et al. 2003; Salem et al. 2005a; Bower et al. 2008; Salem et al. 2007). Nevertheless, there has been little research on how fish diets affect the expression of genes that participate in growth. Chapalamadugu et al. (2009) evaluated the influence of dietary carbohydrate level (0, 15, 25, or 35%) on the temporal mRNA expression patterns (4, 8 or 12 week) of transcription factors that regulate satellite cell myocyte addition (MA) in rainbow trout. They showed that 15% and 25% carbohydrate containing diets significantly up-regulate *Myod* and *Myf5*, but not *Pax7* after 12 weeks of feeding. The high carbohydrate diet (35%) attenuated the increased mRNA expression of these transcription factors. These results suggest a potential role for satellite cells in nutrient sensing ability in fish. Recently Alami-Durante et al. (2010a) observed expression of related genes in white and red muscle of rainbow trout fed with graded levels of a mix of plant protein sources as substitutes for fishmeal. They showed changes in the expression of genes involved in lysosomal proteolysis in the white muscle. In another study Alami-Durante et al. (2010 b) identified the amino acid profile and the changes in expression of myogenic regulatory factor genes and

of myosin heavy chains in the muscle of juvenile rainbow trout when fed plant protein sources diets. They did not observe changes in the expression of genes *MyoD*, *MyoD2* or *myogenin* in lateral red muscle. However, they have demonstrated changes in the expression of the *MyoD* and of the fast-MHC genes in skeletal white muscle. These results indicate that the white and red muscles of rainbow trout are differently affected by nutritional changes.

Two studies in zebrafish have been performed with nutritional genomics. Robison et al. (2008) identified sexual dimorphism in hepatic gene expression in response to different percentages of carbohydrates in diets (0%, 15%, 25% and 35%) from the larval stage through sexual maturity, using microarrays and qRT-PCR. They observed substantial sexual dimorphism in the hepatic transcriptome-like genes associated with oxidative metabolism, carbohydrate metabolism and energy production, among others. Drew et al. (2008) identified metabolic pathways regulated by starvation in the liver and in the brain of zebrafish, using microarray and quantitative real-time PCR (qRT-PCR) analyses in adult females after 21 days of starvation. They observed that starvation regulated gene expression of components of metabolic activity, reduced lipid metabolism, protein biosynthesis, caused proteolysis and cellular respiration. The response of the zebrafish hepatic transcriptome to starvation was strikingly similar to that of rainbow trout, and less similar to mouse, while the response of common carp differed considerably from the other three species. These results support the use of this fish as a model organism for nutritional genomic studies, where the results of these studies could be compared to similar question in aquacultured fish.

2.13. Conclusions and Future Perspectives

Zebrafish have several advantages that allow it to be used as a model organism for nutritional and growth studies, especially for nutritional genomic studies. Zebrafish possess the most developed genomic program compared to that of any other aquacultured fish, they are easy to maintain and breed, have short generational time and produce a large number of offspring**.** Moreover, the zebrafish is evolutionally closer to the most important aquacultured fish than any other mammalian model organism, including the mouse. However, zebrafish can feed from animal and vegetal protein sources, which allows us to infer that it has nutritional pathways similar to those of cultivated herbivores such as carp and tilapia, and to those of carnivores such as trout and salmon. Nevertheless, more studies on zebrafish nutrition and protein, carbohydrates and lipid requirements are needed to assess the nutritional requirement for optimal skeletal muscle growth in fishes. New possible areas of investigation in zebrafish are needed in nutrition and nutrigenomics. Aquaculture investigation must take advantages of and find answers in model organisms such as the zebrafish, where studies of gene expression and genetic variants in response to nutrients could potentially be translational to aquacultured species. Studies in cultured species have been

a major limitation because they require crosses to observe segregation of the different genetic variants, and the majority of these cultured fishes have a long generation time. In this case, the use of zebrafish as a model may be a good starting point to look for the genetic variants present in many genes. These genetic variants could be responsible for phenotypic variability of economically important traits such as growth. The identification of polymorphic sites in coding genes is a step forward to future investigations on any associations of these variants with the increase or reduction in the phenotypic expression of productive traits.

Acknowledgments

This work was supported by the following fellowships to PU: Conicyt AT-24091052 and Doctoral fellowship from the Consorcio Empresarial de Genética y Desarrollo Biotecnológico para la Industria Salmonera (Aquainnovo S.A.). The authors acknowledge the help of Daniela Romo for your assistance in photography and Francisco Estay (Piscicola Hulilco Ltda.) for providing gametes, fertilization and rearing protocols for rainbow trout. PU also wishes to thank the Programa de Doctorado en Ciencias de Recursos Naturales of the Universidad de La Frontera. We are especially grateful to Rashida Lathan for the English editing in the final version of this work.

2.14. References

Accini G (2009) Estimación de heredabilidad e identificación de marcadores RAPD asociados a tasa de crecimiento en zebrafish (*Danio rerio*). Tesis de Ingeniero Agrónomo, Facultad de Ciencias Agronómicas, Universidad de Chile, 33 pp

Acosta J, Carpio Y, Borroto I, Gonzalez O, Estrada M (2005) Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol 119:324–331

Albertson R, Payne-Ferreira T, Postlethwait J, Yelick P (2005) Zebrafish *acvr2a* and *acvr2b* exhibit distinct roles in craniofacial development. Dev Dyn 233:1405–1418

Aleström P, Holter J, Nourizadeh-Lillabadi R (2006) Zebrafish in functional genomics and aquatic biomedicine. Trends Biotechnol 24:15-21

Alami-Durante H, Médale F, Cluzeaud M, Kaushik S (2010a) Skeletal muscle growth dynamics and expression of related genes in white and red muscle of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture 303:50-58

Alami-Durante H, Wrutniak-Cabello C, Kaushik SJ, Médale F (2010b) Skeletal muscle cellularuty and expression of myogenic regulatory factors and myosin heavy chains in rainbow trout (*Oncorhynchus mykiss*):Effects of changes in dietary plant protein sources and amino acid profiles. Comp Biochem Physiol A 156:561-568

Allendorf F, Thorgaard G (1984) Tetraploidy and the evolution of salmonid fishes. In: Turner B (ed) Evolutionary Genetics of Fishes. Plenum Press, New York, pp 1-46

Aparicio S, Chapman J, Stupka E, Putnam N, Chia J, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Sollewinjn Gelpke M, Roach J, Oh T, Ho IY, Wong M, Detter Ch, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith S, Clark M, Edwards YJK, Doggett N, Zharkikh A, Tavtigian S, Pruss D; Barnstead M, Evans Ch, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan H, Elgar G, Hawkins T, Venkatesh B, Rokhsar D, Brenner S (2002) Whole-Genome Shotgun Assembly and Analysis of the Genome of *Fugu rubripes*. Science 297:1301-1310

Araneda C, Neira R, Lam N, Iturra P (2008). Salmonids. In: Kocher TD, Kole C (eds) Genome mapping and genomics in fishes and aquatic animals, Springer-Verlag, Berlin, Heidelberg, pp 1-43

Atchley W, Fitch W, Bronnerfraser M (1994) Molecular evolution of the Myod family of transcription factors. Proc Natl Acad Sci USA 91: 11522–11526

Barbazuk W, Korf I, Kadavi I, Heyen J, Tate S, Wun E, Bedell J, McPherson J, Johnson S (2000) The syntenic relationship of the zebrafish and human genomes. Genome Res 10:1351-1358

Bell M (2001) Lateral plate evolution in the threespine stickleback: getting nowhere fast. Genetica 1: 445-461

Bergeron S, Milla L, Villegas, R, Shen M-C, Burgess S, Allende M, Palma V, Karlstrom R (2008) Expression profiling identifies novel Hh/Gli regulated genes in developing zebrafish embryos. Genomics 91:165-177

Biga P, Goetz F (2006) Zebrafish and giant danio as models for muscle growth: determinate versus indeterminate growth as determined by morphometric analysis. Am J Physiol Regul Integr Comp Physiol 291: 1327–1337

Boulding EG, Culling M, Glebe B, Berg PR, Lien S, Moen T (2008) Conservation genomics of Atlantic salmon: SNPs associated with QTLs for adaptative traits in parr from four trans-Atlantic backcrosses. Heredity 101:381-391

Bower N, Li X, Taylor R, Johnston A (2008) Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. J Exp Biol 211: 3859-3870

Bradley K, Elmore B, Breyer J, Yaspan B, Jessen J, Knapik E, Smith J (2007) A major zebrafish polymorphism resource for genetic mapping. Genome Biol 8: 55-65

Breder C, Rosen D (1966) Modes of reproduction in Fishes. The Natural History Press, New York, pp 941

Briggs J (2002) The zebrafish: A new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol 282:R3-R9.

Brown M (1957) Experimental studies on growth. In Brown M (ed) The physiology of fishes. Academmic Press, New York, pp 261-400

Carpio Y, Acosta J, Morales R, Santisteban Y, Sanchéz A, Estrada M (2009) Regulation of body mass growth through activin type IIB receptor in teleosts fish. Gen Comp Endocrinol 160:158-167

Carvalho A, Sá R, Oliva-Teles A, Bergot P (2004) Solubility and peptide profile affect the utilization of dietary protein by common carp (*Cuprinus carpio*) during early larval stages. Aquaculture 234:319-333

Carvalho A, Araujo L, Santos M (2006) Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical, and a purified starter diet on larval performance. Aquacult Res 37:1107–1111

Cenadelli S, Maran V, Bongioni G, Fusetti L, Parma P, Aleandri R (2007) Identification of nuclear SNPs in gilthead seabream. J. Fish Biol. 70:399-405

Cossins A, Crawford D (2005) Fish as model for environmental genomics. Nat. Rev. Genet. 6:324-340

Cowey C, Sargent J (1976) Lipid nutrition in fish. Comp Biochem Physiol 57B: 269-273

Clark M (2003) Genomics and mapping of teleostei. Comp Funct Genomics 4:182-193

Cresko W, McGuigan K, Phillips P, Postlethwait J (2007) Studies of threespine stickleback developmental evolution: progress and promise. Genetica 129:105-126

Chapalamadugu K, Robison B, Drew R, Powell M, Hill R, Amberg J, Rodnick K, Hardy R, Hill M, Murdoch G (2009) Dietary carbohydrate level affects transcription factor expression that regulates skeletal muscle myogenesis in rainbow trout. Comp. Biochem. Physiol. B 153: 66-72

Chauvigné F, Gabillard J, Weil C, Rescan P (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6 and myostatin mRNA expression in rainbow trout myotomal muscle. Gen Comp Endocrinol 132: 209–215

Chen Y, LeeW, Liu C, Tsai H (2001) Molecular structure, dynamic expression, and promoter analysis of zebrafish (*Danio rerio*) Myf-5 gene. Genesis 29: 22–35

Chen F, Lee Y, Jiang Y, Wang S, Peatman E, Abernathy J, Liu H, Liu SK, Kucuktas H, Ke CH, Liu Z (2010) Identification and characterization of Full-Length cDNAs in Channel Catfish (*Ictalurus punctatus*) and Blue Catfish (*Ictalurus furcatus*). Plos One 12 e 11543

Crollius H, Weissenbach J (2008) Fish genomics and biology. Genome Res 15:1675-1682

Daga R, Thode G and Amores A (1996) Chromosome complement, C-banding, Ag-NOR and replication banding in the zebrafish *Danio rerio*. Chromosome Res. 4:29-32

Davidson W, Koop B, Jones S, Iturra P, Vidal R, Maass A, Jonassen I, Lien S, Omholt S (2010) Sequencing the genome of the Atlantic salmon (*Salmo salar*). Genome Biol. 11:403

Davis GP, Hetzel DJ (2000) Integrating molecular genetics technology with traditional approaches for genetic improvement in aquaculture species. Aquacult Res 31:3-10

Dabrowski K, Hliwa P, Gomulka P, Sienicki M, Carvalho AP, Ostaszewska T, Terjesen B (2005) Studies on the utilization of free amino acid, casein-based, or commercial diets in rearing common carp larvae. Abstr of Aquac Am 344, New Orleans, USA.

Dahm R (2002) Atlas of embryonic stages of development in the zebrafish. In: Nusslein-Volhard C, Dahm R (eds) Zebrafish: A Practical Approach, Oxford University Press, Oxford, 219–236 pp

Dahm R, Geisler R (2006) Learning from small fry: The zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol 0:1-17

De-Santis C and Jerry D (2007) Candidate growth genes in finfish- Where should we be looking? Aquaculture 272: 22-38

Dominik S, Henshall J, Kube P, King H, Lien S, Kent M, Elliott N (2010) Evaluation of an Atlantic salmon SNP chip as a genomic tool for the application in a Tasmanian Atlantic salmon (*Salmo salar*) breeding population. Aquaculture 308: S56-S61

Douglas, S (2006) Microarray Studies of Genes Expression in Fish. J Integr Biol 10 (4): 474-489

Du Sh (2004) Molecular Regulation of Fish Muscle Development and Growth In: Gong Z, Korzh V (eds) Molecular Aspects of Fish and Marine Biology, volume 2, Fish Development and Genetics The Zebrafish and Medaka Models. World Scientific, Singapure pp 581-611

Dutta, S (1993) Food and feeding habits of *Danio rerio* (Ham. Buch.) inhabiting gadigarh stream, Jammu. J Freshw Biol 5:165–168

Dumas A, Frane J, Bureau D (2010) Modelling growth and body composition in fish nutrition: Where have we been and where are we going? Aquacult Res 41:161-181

Drew R, Rodnick K, Settles M, Wacyk J, Churchill E, Powell M, Hardy R, Murdoch G, Hill R, and Barrie R (2008). Effect of starvation on the transcriptomes of the brain and liver in adult female zebrafish. Physiol Genomics 35:283-295

Driever W, Stemple D, Schier A, Solnica-Krezel L (1994) Zebrafish: genetic tools for studying vertebrate development. Trends Genet 10:152-159

Eaton R, Farley R (1974a). Spawning Cycle and Egg Production of Zebrafish, *Brachydanio rerio*, in the Laboratory. Copeia 1:195-204

Eaton R, Farley R (1974b) Growth and the reduction of depensation of zebrafish, *Brachedanio rerio* reared in the laboratory. Copeia 1: 204-209

Ehrlich J, Sankoff D, Nadeau J (1997) Synteny conservation and chromosome rearrangements during mammalian evolution. Genetics 147:289-296

Erbay E, Park I, Nuzzi P, Schoenherr C, Chen J (2003) IGF-II transcription in skeletal myogenesis is controlled by *mTOR* and nutrients. J Cell Biol 163: 931–936

Estay F, Cerisola H, Téllez V (1994) Biologia del desarrollo y reproducción artificial en la trucha arcoíris. Publicación del grupo de investigación de la Universidad de Chile, Universidad Católica de Valparaíso y del Instituto Fomento Pesquero, Chile, pp1-28

Falconer D, MacKay T (1996) Introduction to quantitative genetics. Longman Group Ltda., Malasia. 464 pp

Fiogbé E, Kestemont P (1995) An assessment of the protein and amino acid requirement in goldfish (*Carassius auratus*) larvae. J Appl Ichthyol 11:282–289

Franch R, Louro B, Tsalavouta M, Chatziplis D, Tsigenopoulos S, Sorropoulou E, Antonello J, Magoulas A, Mylonas C, Babbucci M, Patarnello T, Power D, Kotoulas G, Bargelloni L (2006) A genetic Linkage Map of the Hermaphrodite Teleost Fish *Sparus aurata* L. Genetics 174: 851-861

Froystad M, Lilleeng E, Bakke-Mckellep A, Vekterud K, Hemre G, Krogdahl A (2008) Gene expression in distal intestine of Atlantic salmon (*Salmo salar* L.) fed genetically modified soybean meal. Aquacult Nutr 14: 204-214

Froystad M, Lilleeng E, Bakke-Mckellep A, Vekterud K, Valen E, Hemre G, Krogdahl A (2009) Distal intestinal gene expression in Atlantic salmon (*Salmo salar* L.) fed genetically modified maize. Aquacult Nutr 15:104-115

German D, Horn M (2006) Gut length and mass in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Mar Biol 148:1123-1134

Goolish E, Okutake K, Lesure S (1999) Growth and Survivorship of larval zebrafish *Danio rerio* on processed diets. N Am J Aquacult 61: 189–198

Goll D, Thompson V, Li H, Wei W, Cong J (2003) The calpain system. Physiol Rev 83: 731–801

Gornung E, Gabrielli I, Cataudella S and Sola L (1997) CMA_3- banding pattern and fluorescence in situ hybridization with 18S rRNA genes in zebrafish chromosomes. Chromosome Res. 5:40-46

Govoroun M, Le Gac F, Guiguen Y (2006) Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalized rainbow trout cDNA libraries. BCM Genomics 7:196

Grunwald D, Eisen J (2002) Headwaters of the zebrafish- emergence of a new model vertebrate. Nat Rev Genet 3:717-724

Hardy R (2010) Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult Res 41: 770-776

Hahlbeck E, Katsiadaki I, Mayer I, Adolfsson-Erici M, James J, Bengtsson B (2004) The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II-kidney hypertrophy, vitellogenin and spiggin induction. Aquat. Toxicol. 70:311-326

Hayes B, Laerdahl JK, Lien S, Moen T, Berg P, Hindar K, Davison WS, Koop BF, Adzhubei A, Hoyheim B (2007) an extensive resources of single nucleotide polymorphism markers associated with Atlantic salmon (*Salmo salar*) expressed sequences. Aquaculture 265:82-90

Hedges S (2002) The origin and evolution of model organisms. Nat. Rev. Genet. 3:838-843

Helterline D, Garikipati D, Stenkamp D, Rodgers D (2006) Embryonic and tissue-specific regulation of myostatin-1 and-2 gene expression in zebrafish. Gen Comp Endocrinol 151(1):90-97

Hinegardner R, Rosen DE (1972) Cellular DNA content and the evolution of teleostean fishes. Am. Nat. 106: 621-644

Hinits Y, Osborn D, Carvajal J, Rigby P, Hughes M (2007) *Mrf4* (*myf6*) is dynamically expressed in differentiated zebrafish skeletal muscle. Gene Expression Patterns 7:738-745

Horn M, Gawlicka A, German D, Logothetis E, Cavanagh J, Boyle K (2006) Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory. Mar Biol 149:1237-1245

Huising M, Geven E, Kruiswijk C, Nabuurs S, Stolte E, Spanings F, Verburg-van Kemenade B, Flik G (2006) Increased leptin expression in common carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. Endocrinology 147:5786–5797

Janvier P (1996) Early Vertebrates. Oxford Science Publications, Clarendon Press, New York, 375 pp

Johansen K, Overturf K (2005) Sequence, conservation, and quantitative expression of rainbow trout Myf-5. Comp Biochem Physiol B: Biochem Mol Biol 140:533–541

Johansen S, Coucheron D, Andreassen M, Ove Karlsen B, Furmanek T, Jorgensen T, Emblem A, Breines R, Nordeide J, Moun T, Nederbragt A, Stenseth N, Jakobsen K (2009) Large-scale sequence analyses of Atlantic cod. New Biotechnol. 25:263-271

Johnston I (1999) Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177:99-115

Johnson R, Johnson T, Londraville R (2000) Evidence for leptin expression in fishes. J Exp Zool A: Comp Exp Biol 286: 718–724

Johnston I, Macqueen D, Watabe S (2008) Molecular Biotechnology of Development and Growth in Fish Muscle. In: Tsukamoto K., Kawamura T, Takeuchi T, Douglas Beard T, Kaiser M (eds) Fisheries for Global Welfare and Environment, 5th World Fisheries Congress, Scotland, pp 241-262

Kaput J, Raymond L and Rodriguez (2003) Nutritional Genomics: the next frontier in the postgenomic era. Physiol Genomics 16:166-177

Kasahara M, Naruse K, Sasaki Sh, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shamida A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa Sh, Shimizu N, Hashimoto Sh, Yang Jun, Lee Y, Matsushima K, Sugano S, Sakaisumi M, Takanori N, Ohishi K, Haga Sh, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita Sh, Kohara Y (2007) The medaka draft genome and insigts into vertebrate genome evolution. Nature 447:714-719

Katsiadaki I, Scott AP, Mayer I (2002) The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. Mar. Environ. Res. 54:725-728

Kimmel C, Ballard W, Kimmel S, Ullmann B, Schilling T (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310

Kobiyama A, Nihei Y, Hirayama Y, Kikuchi K, Suetake H, Johnston I, Watabe S (1998) Molecular cloning and developmental expression patterns of the MyoD and MEF2 families of muscle transcription factors in the carp. J Exp Biol 201:2801–2813

Kurokawa T, Uji S, Suzuki T (2005a) Identification of pepsinogen gene in the genome of stomachless fish, *Takifugu rubripes* Comp Biochem and Physiol Part B: Biochem Mol Biol 140: 133-140

Kurokawa T, Uji S, Suzuki T (2005b) Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides 26: 745–750

Lauder G, Liem K (1989) The evolution and interrelationships of the Actinopterygian fishes. Bull Mus Comp Zool 150: 95–187

Lawrence C (2007) The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture 269:1-20

Lee S, McPherron A (2001) Regulation of myostatin activity and muscle growth. PNAS 16: 9306-9311

Leong J, Jantzen S, Von Schalburg K, Cooper G, Messmer A, Liao N, Munro S, Moore R, Holt R, Jones S, Davison W, Koop B (2010) *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. BCM Genomics 11:279

Li P, Peatman E, Wang Sh, Feng J, He Ch, Baoprsertkul P, Xu P, Kucuktas H, Nandi S, Somridhivej B, Serapion J, Simmons M, Turan C, Liu L, Muir W, Dunham R, Brady Y, Grizzle J, Liu Z (2007) Towards the ictalurid catfish transcriptome: generation and analysis of 31,215 catfish ESTs. BCM Genomics 8:117

Lilleeng E, Froystad M, Ostby G, Valen E, Krogdahl A (2007) Effects of diet containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (*Salmo salar* L.). Comp Biochem Physiol Part A: Mol Integr Physiol 147: 25-36

Liu H, Jiang Y, Wang Sh, Ninwichian P, Somridhivej B, Xu P, Abernathy J, Kucuktas H, Liu Z (2009) Comparative analysis of catfish BAC end sequences with the zebrafish genome. BCM Genomics 10:592

Liu Z, Cordes J (2004) DNA marker technologies and their applicationsin aquaculture genetics. Aquaculture 238:1-37

Lochman R, Phillips H (1996) Nutrition and feeding of baitfish. Aquacult Mag 4: 87–89

Lo Pestri R, Lisa C, Di Stasio L (2009) Molecular genetics in Aquaculture. Ital J of Anim Sci 8: 299-313

Lu J, Peatman E, yang q, Wang Sh, Hu Z, Reecy J, Kucuktas H, Liu Z (2010) The catfish genome database cBARBEL: an informatics platform for genome biology of ictalurid catfish. Nucleic Acids Res. 39:D815-D821

Maccatrozzo L, Bargelloni L, Cardazzo B, Rizzo G, Patarnello T (2001) A novel second myostatin gene is present in teleost fish. FEBS Lett 509: 36–40

Macqueen D, Johnston I (2008) Evolution of follistatin in teleosts revealed through phylogenetic, genomic and expression analyses. Dev Genes Evol 218:1-14

Marti M, Moreno-Aliaga M, Zulet A, Martinez J (2005) Avances en nutrición molecular: nutrigenómica y nutrigenética. Nutr Hosp 3: 157-164

McClure M, McIntyre P, McCune A (2006) Notes on the natural diet and habitat of eight danioin fishes, including the zebrafish *Danio rerio*. J Fish Biol 69:553–570

McPherron A, Lawler A, Lee S (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387: 83–90

Meinelt T, Schultz C,Worth M, Kurzinger H, Steinberg C (1999) Dietary fatty acid composition influences the fertilization rate of zebrafish (*Danio rerio* Hamilton-Buchanon). J Appl Ichthyol 15: 19–23

Meinelt T, Schultz C, Worth M, Kurzinger H, Steinberg C (2000) Correlation of diets high in n6-polyunsaturated fatty acids with high growth rate in zebrafish (*Danio rerio*). Comp Med 50: 43–45

Metscher B, Ahlberg P (1999) Zebrafish in Context: Uses of a Laboraty Model in Comparative Studies. Dev Biol 210: 1-4

Meli R, Prasad A, Patowary A, Lalwani K, Maini J, Sharma M, Singh A, Kumar G, Jadhav V, Scaria V and Sivasubbu S (2008) FishMap: A Community Resource for Zebrafish Genomics. Zebrafish 5:125-130

Moen T, Hayes B, Baranski M, Berg P, Kjoglum S, Koop B, Davidson W, Omholt S, Lien S (2008a) A linkage map of the Atlantic salmon (*Salmo salar*) based on EST-derived SNP markers. BCM Genomics 9:223-237

Moen T, Hayes B, Nilsen F, Delghandi M, Fjalestad K, Fevolden SE, Berg PR, Lien S (2008b) Identification and characterization of novel SNP markers in Atlantic cod: Evidence for directional selection. BMC Genetics 9:18

Mommsen T (2001) Paradigms of growth in fish. Comp. Biochem. Physiol. B 129:207-219

Moriyama S, Ayson F, Kawauchi H (2000) Growth Regulation by Insulin-like Growth Factor-I in Fish. Biosci Biotechnol Biochem 64 (8): 1553-1562

Moyle P, Cech J (2000) Fishes. An Introduction to Ichthyology. Prentice Hall Inc., USA, 612pp.

Mutch M, Wahli W, Williamson G (2005) Nutrigenomics and nutrigenetics: the emerging faces of nutrition. The FASEB J 19: 1602-1615

Müller M, Kersten S (2003) Nutrigenomics: goals and strategies. Nat Rev Genet 4:325 -322

Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) A Medaka Gen Map: The Trace of Ancestral Vertebrate Proto-Chromosomes Revealed by Comparative Gene Mapping. Genome Res. 14:820-828

Nielsen E, Hansen M, Meldrup D (2006) Evidence of microsatellite hitch-hiking selection in Atlantic cod (*Gadus morhua* L.): implications for inferring population structure in nonmodel organisms. Mol. Ecol. 15:3219-3229

NRC National Research Council (1993) Nutrient Requeriments of coldwater fishes. National Academy of Science, Washington, DC USA, 63 pp

Ojima Y (1983) Fish Cytogenetics. Suiko-sha, Tokyo, Japan, 453 pp

Orban L, Wu Q (2008) Cyprinids In: Kocher TD, Kole C (eds) Genome mapping and genomics in fishes and aquatic animal, volume 2, Springer-Verlag, Berlin, Heidelberg, pp 45-97

Ostbye T, Bardal T, Vegusdal A, Frang O, Kjorsvik E, Andersen O (2007) Molecular cloning of the Atlantic salmon activin receptor IIB cDNA-Localization of the receptor and myostatin in vivo and in vitro in muscle cells. Comp Biochem Physiol 2: 101–111

Panserat S, Ducasse-Cabanot S, Plagnes-Juan E, Srivastava P, Kolditz C, Piumi F, Esquerré D, Kaushik S (2008) Dietary fat level modifies the expression of hepatic genes in juvenile rainbow trout (*Oncorhynchus mykiss*) as revealed by microarray analysis. Aquaculture 275: 235-241

Panserat S, Kirchner S and Kaushik S (2007) Nutrigenomics. In: Nakagawa H, Sato M, Gatlin III D (eds) Dietary supplements for the health and quality of cultured fish, CAB International North America, pp 210-229

Panserat S, Kaushik S (2010) Regulation of gene expression by nutritional factors in fish. Aquacult Res 41: 751-762

Postlethwait J (2004) Evolution of the zebrafish genome. In: Gong Z, Korzh V (eds) Molecular Aspects of Fish and Marine Biology, volume 2, Fish Development and Genetics The Zebrafish and Medaka Models. World Scientific, Singapure pp 581-611

Phillips D, Krestor D (1998) Follistatin: a multifunctional regulatory protein. Front Neuroendocrinol 19: 287–322

Quinn N, Levenkova N, Chow W, Bouffard P, Boroevich K, Knight J, Jarvie T, Lubieniecki K, Desany B, Koop B, Harkins T, Davidson W (2008) Assessing the feasibility of GS FLX Pyrosequencing for sequencing the Atlantic salmon genome. BMC Genomics 9:404

Reid DP, Santo A, Glebe B, Danzmann RG, Ferguson M (2005) QTL for body weight and condition factor in Atlantc salmon (*Salmo salar*): comparative analysis with rainbow trout (*Oncorhynchus mykiss*) and Artic charr (*Salvelinus alpinus*). Heredity 94:166-172

Rebbapragada A, Benchabane H, Wrana J, Celeste A, Attisano L (2003) Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230–7242

Rexroad III C, Rodriguez M, Coulibaly I, Gharbi K, Danzmann R, DeKoning J, Phillips R, Palti Y (2005) Comparative mapping of expressed sequence tags containing microsatellites in rainbow trout (*Oncorhynchus mykiss*). BCM Genomics 6:54-62

Rexroad III C, Palti Y, Gahr S, Vallejo R (2008) A second generation genetic map for rainbow trout (*Oncorhynchus mykiss*). BCM Genet 9:74-88

Rexroad III CE, Lee Y, Keele JW, Karamycheva S, Brown G, Koop B, Gahr SA, Palti Y, Quackenbush J (2003) Sequence analysis of a rainbow trout cDNA library and creation of a gene index. Cytogenet Genome Res 102:347-354

Ryynänen HJ and Primmer Craig (2006) Single nucleotide polymorphism (SNP) discovery in duplicated genomes: intron-primed exon crossing (IPEC) as a strategy for avoiding amplification of duplicated loci in Atlantc salmon (*salmo salar*) and other salmon fishes. BCM Genomics 7: 192

Ricker, W (1958) Hanbook of computations for biological statistics of fish populations. Bull Fish Res Bd Canada, 300 pp

Robison B, Drew R, Murdoch G, Powell M, Rodnick K, Settles M, Stone D, Churchill E, Hill R, Papasani M, Lewis S, Hardy R (2008) Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*). Comp Bichem and Physiol Part D 2:141-154

Roush W (1996) Zebrafish embryology builds a better model vertebrate. Science 272:1103

Rust M (2002) Nutritional Physiology. In: Halver J, Hardy R (eds) Fish Nutrition, third edition, academic press, USA, pp 368-446

Salem M, Nath J, Rexroad C, Killefer J, Yao J (2005a) Identification and molecular characterization of the rainbow trout calpains (*Capn1* and *Calp2*): their expression in muscle wasting during starvation. Comp Biochem Physiol B:Biochem Mol Biol 140: 63-71

Salem M, Yao J, Rexroad C, Kenney P, Semmens K, Killefer J, Nath J (2005b) Characterization of calpastatin gene in fish: Its potential role in muscle growth and fillet quality. Comp Biochem and Physiol B: Biochem Mol Biol 141: 488-497

Salem M, Silverstein J, Rexroad III C, Yao J (2007) Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). BCM Genomics 8:328-344

Santigosa E, Sánchez J, médale F, Kaushik S, Peréz-Sánchez J, Gallardo M (2008) Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture 282: 68-74

Santos M, Micael J, Carvalho A, Morabito R, Booy P, Massanisso P, Lamoree M, Reis-Henriques M (2006) Estrogens counteract the masculinizing efect of tributyltin in zebrafish. Comp Biochem and Physiol C:Toxicol Pharmacol 142:151-155

Sarropoulou E, Power DM, Magoulas A, Geisler R, Kotoulas G (2005) Comparative analysis and characterization of expressed sequence tags in gilthead sea bream (*Sparus aurata*) liver and embryos. Aquaculture 243:69-81

Sarropoulou E, Franch R, Louro B, Power D, Bargelloni L, Magoulas An, Senger F, Tsalavouta M, Patarnello T, Galibert F, Kotoulas G, Geisler R (2007) A gene-based radiation hybrid map of the gilthead sea bream *Sparus aurata* refines and exploits conserved synteny with *Tetraodon nigroviridis*. BCM Genomics 8:44

Seiliez I, Gabillard J, Skiba-Cassy S et al (2008) An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (Onchorinchus mykiss). Am J Physiol Regul Integr Comp Physiol 295: 329–335

Senger F, Priat C, Hitte Ch, Sarropoulou E, Franch R, Geisler R, Bargelloni L, Power D, Galibert F (2006) The first radiation hybrid map of perch-like fish: The gilthead seabream (*Sparus aurata* L). Genomics 87:793-800

Smith C, Elfstrom C, Seeb L, Seeb J (2005) Use of sequence data from rainbow trout and Atlantic salmon for SNP detection in Pacific salmon. Mol Ecol 14:4193-4203

Spence R, Fatema M, Ellis S, Ahmed Z, Smith C (2007) Diet, growth and recruitment of wild zebrafish in Bangladesh. J Fish Biol 71:304-309

Stickney H, Schmutz J, Woods I, Holtzer C, Dickson M, Kelly P, Myers R, Talbot W. (2002) Rapid mapping of zebrafish mutations with SNPs and oligonuleotide microarrays. Genome Res 12:1929-1934

Tan X, Hoang L, Du S (2002) Characterization of muscleregulatory genes, Myf5 and myogenin, from striped bass and promoter analysis of muscle-specific expression. Mar Biotechnol 4: 537–545

Tave D (1993) Genetics for fish hatchery managers.An AVI book, New York, 415 pp

Thorsen J, Zhu B, Frengen E, Osoegawa K, Jong PJ, Koop BF, Davidson W, Hoyheim B (2005) A highly redundant BAC library of Atlantic salmon (Salmo salar):an important tool for salmon projects. BCM Genomics 6:50

Fjalestad KT, Moen T, Gomez-Raya L (2003) Prospects for genetic technology in salmon breeding programmes. Aquacult Res 34:397-406

Turchini G, Torstensen B, Ng W-K (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1:10-57

Vignal A, Milan D, San Cristobal M, Eggen (2002) A review on SNP and other types of molecular markers and their use in animal genetics. Genet Sel Evol 34:275-305

Volff J (2005) Genome evolution and biodiversity in teleost fish. Heredity 94:280-294

Von Hertell U, Hörstgen-Schwarrk G, Langholz H, Jung B (1990). Family studies on genetic variability in growth and reproductive performance between and within test fish populations of the zebrafish, *Brachydanio rerio*. Aquaculture 85:307-315

Wang Y, Li C, Lee G, Tsay H, Tsai H, Chen Y (2008) Inactivation of zebrafish mrf4 leads to myofibril misalignment and motor axon growth disorganization. Dev Dyn 237: 1043–1050

Wang S, Peatman E, Abernathy J, Waldbieser G, Lindquist E, Richardson P, Lucas S, Wang M, Li P, Thimmapuram J, Liu L, Vullaganti D, Kucuktas H, Murdock Ch, Small B, Wilson M, Liu H, Jiang Y,

Lee Y, Chen F, Lu J, Wang W, Xu P, Somridhivej B, Baoprasertkul P, Quilang J, Sha Z, Bao B, Wang Y, Wang Q, Takano T, Nandi S, Liu S, Wong L, Kaltenboeck L, Quiniou S, Bengten E, Miller N, Trant J, Rockhsar D, Liu Z, the Catfish Genome Consortium (2010) Assembly of 500,000 inter-specific catfish expressed sequence tags and large scale gene-associated marker development for whole genome association studies. Genome Biol. 11:R8

Watanabe T (1981) Lipid nutrition in fish. Comp Biochem Physiol B 73:3–15

Watanabe T, Kitajima C, Fujita S (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. Aquaculture 34:115-145

Wegrzyn D, Ortubay S (2009) Salmonidos en patagonia, volumen 1, Mar del plata, Argentina 164 pp

Wesmajervi MS, Tafese T, Stenvik J, Fjalested KT, Damsgard B, Delghandi M (2007) Eight new microsatellite markers in Atlantic cod (*Gadus morhua* L.) derived from an enriched genomic library. Mol. Ecol. 7:138-140

Westerfield M (2000) The Zebrafish Book. A guide for the laboratory use of zebrafish (*Danio rerio*), University of Oregon Press, Eugene, Oregon USA, 385 pp

Wittbrodt J, Shima A, Schartl M (2002) Medaka – A model organism from the far east. Nat Rev Genetic 3: 53-64

Woods G, Wilson C, Friedlander B, Chang P, Reyes K, Nix R, Kelly D, Chu F, Postlethwait H, Talbot W (2005) The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res 15:1307-1314

Wright D, Nakamichi R. Krause J. Butlin R (2006) QTL Analysis of Behavioral and Morphological Differentiation Between Wild and Laboratory Zebrafish (*Danio rerio*). Behav Genet 36: 271-284

Xu P, Wang Sh, Liu L, Thorsen J, Kukuktas H, Liu Z (2007) A BAC-based physical map of the channel catfish genome. Genomics 90:380-388

Chapter 3. Growth response and expression of muscle growth-related candidate genes in adult zebrafish (Danio rerio) fed plant and fishmeal protein-based diets.

Paper submitted in Zebrafish
ID: ZEB-2012-0823

Growth response and expression of muscle growth-related candidate genes in adult zebrafish (*Danio rerio*) fed plant and fishmeal protein-based diets.

Pilar E. Ulloa[1,3*], Andrea A. Peña[2], Carla D. Lizama[3], Cristian Araneda[3], Patricia Iturra[4], Roberto Neira[3,5] and Juan F. Medrano[6]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Av. Francisco Salazar 01145, Casilla 54-D, Temuco, Chile.

[2] Laboratorio de Genética e Inmunología Molecular. Pontificia Universidad Católica de Valparaíso. Av. Brasil 2950, Valparaíso, Chile.

[3]Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile.

[4]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70061-7, Santiago, Chile.

[5]AQUAINNOVO S.A., Polpaico 037, Puerto Montt, Chile.

[6]Department of Animal Science, University of California-Davis, One Shields Avenue, CA 95616, USA.

Abstract

The main objective of this study was to examine the effects of a plant protein- vs. a fishmeal-based diet on growth response and expression of growth-related genes in the muscle of adult zebrafish (*Danio rerio*), in a population of 24 families. To control for familiar variation, each family was split to create two fish populations with similar genetic backgrounds. The fish were fed either fishmeal (FM, control diet) or plant protein (PP, experimental diet) as the unique protein source in their diets from 35 to 98 days post fertilization (dpf). Measurements were taken to evaluate growth response to each diet. In order to examine the PP diet's effect on gene expression, individuals from three similar families with average growth in both populations were selected. In order to examine the effect of familiar variation, the three families were evaluated separately. At 98 dpf, eight growth-related genes (*Igf1a, Igf2a, mTOR, Pld1a, Mrf4, Myod, Myogenin* and *Myostatin1b*) were evaluated in males and females. This study demonstrated that PP diet reduced growth as compared with FM diet in both sexes. Expression of *Myogenin* (X5.0 ± 0.67) and *Mrf4* (X3.42 ± 0.56) increased in males in response to PP diet as compared to FM diet, whereas *Igf2a* was

under-expressed (X0.71 ± 0.09). In females, there were no significant effects for any of the eight genes in response to PP diet. The effect of familiar variation on gene expression was observed in males and females. This study showed that plant protein and family variation have important effects on gene expression in fish muscle.

3.1. Introduction

Over the last three decades, worldwide farmed fish production has grown at an average rate of 8.8% per year[1]. However, wild fish must be captured to produce fishmeal and fish oil, the main protein and lipid sources for farmed fish feed. This resource has been overexploited, with wild fish harvests decreasing from 10.7 million tons in 2004 to 4.2 million tons in 2010[1]. Farmed fish production requirements have increased the demand for fishmeal and fish oil, generating a sharp rise in prices of these products and negatively impacting the environment and natural resources[2]. The increased cost reduces the profitability of aquaculture, as feed represents 40-50% of production costs at the farm level and 30-35% of the total cost of the final product[3]. As a result, plant protein sources have been explored as an alternative to animal protein for use in the feed of both omnivorous (tilapia, catfish, carp, milkfish) and carnivorous fish (salmon, trout, tuna)[4]. The plant protein source most commonly incorporated into fish feed has been soy flour. Herbivorous and carnivorous fish diets often contain between 15% and 45% soybean meal. The use of corn, peas, lupins, canola, barley, and wheat has also gradually become more common in feed formulation[5]. If current fish production levels are maintained, it is anticipated that growth in aquaculture will come to depend strongly on plant protein sources over the next 10 to 12 years[1].

These changes in fish nutrition affect multiple physiological functions in farmed fish, which are reflected in important production traits such as growth[6-9]. Some studies in seabream (*Sparus aurata)*, Atlantic salmon (*Salmo salar)* and rainbow trout (*Oncorhynchus mykiss*) reported decreased growth when 50%, 75% or 100% of the fish meal (FM) in the feed was replaced with plant protein (PP) [6, 7, 9]. However, other studies in rainbow trout showed that FM may be replaced partially by soybean without affecting growth[10, 11]. One of the greatest challenges is formulating a more herbivorous feed appropriate for a carnivorous fish (such as the salmonids). In recent years, a new faces of nutrition have allowed integrate nutrition and genomics analysis through nutrigenomics approach, which have added to the understanding of the effect of diet on gene expression[12]. Nutrigenomic studies in farmed fish have addressed the replacement of different percentages of FM with PP in the diet. These studies provide interesting insights into the effects of formulated diets on gene expression, especially in the intestine and liver[13-17]. However, gene expression in

fish muscle has been scarcely documented, despite the fact that this tissue is greatly affected by nutrition and represents about 60% of total fish body weight[6, 18]. A recent study showed that different genotypes of Atlantic salmon fed a vegetable oil-based diet showed specific responses in gene expression patterns in the liver transcriptome[19]. However, nutrigenomics study to clarify the effect of a plant protein-based diet and familiar variation on gene expression related to fish muscle growth has been sparse.

Several genes associated with growth have been mapped and sequenced. These genes are candidates for studies on gene expression in response to diet. Among the possible pathways that regulate fish growth is the somatotropic axis, which includes genes such as those for insulin-like growth factor 1 and 2 (*Igf1* and *Igf2*) and target genes that participate in the signal transduction network (*mTOR*, *Pld1a*). Furthermore, myogenic regulatory factors (MRFs) (including genes such as *Myod*, *Myogenin*, *Mrf4*, *Myf5*) also positively influence fish growth[20-22]. In contrast, *Myostatin* (*Mstn*) acts as a transforming growth factor and negatively influences fish growth[20]. These pathways generate a dynamic balance of positive and negative signals that determine muscular development[23]. *Igf1* is involved in the cell cycle process, cell division, mitosis and protein transport[22]; *Igf2* controls skeletal myogenesis and is involved in the AKT/mTOR pathway (the mammalian target of rapamycin)[24]. *mTOR* contributes to the regulation of protein synthesis in hypertrophy and leads to the production of *Igf2*[25]; *Pld1* (phospholipase D1) participates in cell growth regulation and the activation of *mTOR* through a nutrient-sensing pathway[26]. MRFs are involved in satellite cell activity (the cells that provide the new nuclei required for skeletal muscle during hypertrophic and hyperplasia)[27, 28]; *Mstn* is a key negative regulator of growth and muscle development[29, 30]. Identifying genes modulated by plant nutrients in zebrafish contributes to understanding of physiological and molecular interactions in fish growth, which could shed light on similar processes in aquacultured fish.

We have proposed zebrafish as a model organism for nutritional genomics studies[31]. The advantages of using zebrafish in this area include: 1) Their short generation interval, allowing for performance of growth studies in a shorter time. 2) The ability to control the mating design, as in a fish farm breeding program. 3) Studies can be conducted with a greater number of fish, ideal for a powerful data analysis to evaluate quantitative traits. 4) Laboratory rearing conditions are more homogeneous and reproducible than farm rearing conditions. 5) Zebrafish are omnivores; they can eat a great variety of foods including FM- and PP-based diets. 6) Finally, this species has ample genomics information available for application in nutritional genomics studies.

The main objective of this study was to examine growth response and evaluate the expression of growth-related genes (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Mstn1b*) in the muscle of adult zebrafish, from a population of 24 experimental families fed with plant and fishmeal protein-based diets.

3.2. Materials and Methods

3.2.1. Experimental design

A zebrafish population of 24 experimental families (single pair mating from no related wild-type strains) was generated in order to examine growth response in a population with wide genetic variability. Embryonated eggs were incubated at 28° ± 1°C for 7 days until hatching in 9 cm diameter Petri dishes (~100 eggs per Petri dish), according to Brand et al.[32] From 7 to 28 dpf (first step, larval rearing) each family was reared separately in an aerated 4L container with a density of 25 $cm^3 \cdot fish^{-1}$. Larvae were reared under optimum physical and chemical water parameters ($25 \leq T\ °C \leq 28.5$, $7 \leq pH \leq 8$; hardness > 100 mg $CaCO_3 \cdot L^{-1}$) and with a photoperiod of 14 h light:10 h dark[32] (Anexo 1). Larvae were fed *ad libitum* three times daily (at 9:30, 13:30 and 17:30 h) with a commercial diet: fine powder feed Sera Micron® (50.2% crude protein, 8.1% crude fat, 4.2% crude fiber and 11.9% ash) and Gold Protein® Micro (49% crude protein, 8% crude fat, 3% crude fiber and 10.5% ash).

At 30 dpf each family was split to generate two replicates (40 fish per family replicate) and create two populations of fish with similar genetic backgrounds. Forty-eight fish groups were randomly distributed into 48 tanks with 14 L capacity (13 x 22 x 48.5 cm). The fish were kept at a density of 350 $cm^3 \cdot fish^{-1}$ [33] with recirculated water under optimum physical and chemical water parameters (Anexo 1). The fish were acclimatized to the new system for 5 days and fed the same commercial diet. Starting on 35 dpf, the first replicate of 24 families was fed a diet containing 100% animal protein as the unique protein source (FM control diet) and the second replicate a diet containing 100% plant protein as the unique protein source (PP experimental diet). The fish were fed *ad libitum* three times daily, six days per week, from larval transition (35 dpf) to sexual maturity (98 dpf) (Figure 3.1).

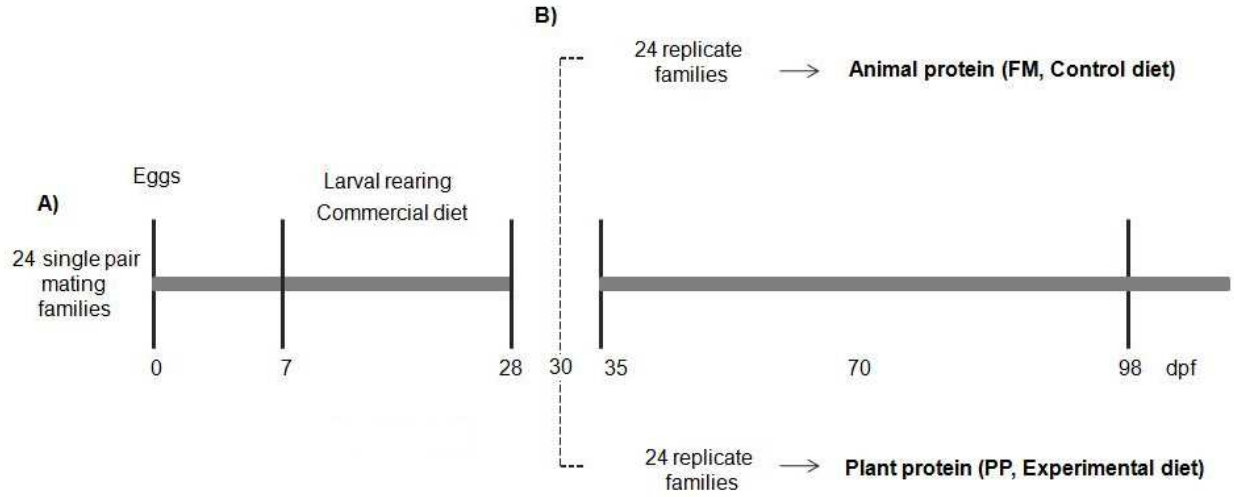

Figure 3.1. Experimental design. A) Basal population of 24 single pair mating families at day zero B) Larval rearing from 7 to 28 days post fertilization (dpf) fed with commercial diet C) At 30 dpf the 24 families were split to generate two replicates of each family (40 fish per family replicate) and create two populations of fish with the same genetic background. At 35 dpf, the first replicate of 24 families was fed with a diet that contained animal protein as a unique protein source (FM, control diet) and the other replicate of families was fed with a diet that contained plant protein as the unique protein source (PP, experimental diet) until 98 dpf.

3.2.2. Experimental diets

Two experimental diets were formulated (Table 3.1). The control diet was formulated to contain fishmeal as the primary protein source and the experimental diet was formulated to contain soy protein concentrate as the primary protein source. Fish oil was included in both diets. Total lipid level varied between diets (11.5% vs. 7.1%) due to differences in lipid content of the two protein sources. However, both diets contained similar proportions of crude protein (56–57% dry matter, DM) and similar energy (391–398 cal·kg^{-1} DM), values fulfilling the nutritional requirements of zebrafish[34, 35].

The diets were manufactured by a twin screw extruder at 130°C (Clextral BC 21, USA), produced as 2 mm diameter pellets and vacuum packed in fish oil by a Dinnissen 10VC machine. The pellets were subsequently crumbled and screened to appropriate particle size (350–500 µm). Proximal chemical analysis of the diet was performed according to the following procedures: dry matter was obtained in an oven at 105°C for 24 h, ash by combustion at 450°C for 16 h, protein (N x 6.25) by the Kjeldahl method, fat after dicholoromethane extraction by the Soxhlet method and gross energy by caloric factor (4, 9, and 4

for protein, lipid and carbohydrate, respectively). Amino acid profiles in diets were determined by liquid chromatography using UV detection, ID-103/AA method[36].

Table 3.1. Ingredients in formulation and nutrient composition in the experimental diets.

Diet	FM	PP
Ingredients ($g \cdot Kg^{-1}$):		
Fishmeal	0.400	0
Feather meal	0.150	0
Powder milk	0.050	0
Poultry by-product meal	0.200	0
Corn gluten meal	0	0.150
Wheat gluten	0	0.350
Soy protein concentrate	0	0.270
Raw starch	0.070	0.075
Fish oil	0.075	0.085
Vitamin/mineral premix[1]	0.050	0.040
Choline chloride	0.005	0.005
Lysine	0	0.005
Ca(H2PO4)2	0	0.020
Total	1	1
Analytical composition (dry bases):		
Dry matter (%)	96.9	95.2
Digestible protein (%)	56.4	57.8
Digestible lipids (%)	11.5	7.10
Starch (%)	17.3	22.5
Ash (%)	12.3	6.2
Gross energy ($cal \cdot kg^{-1}$)	398	391
Analyzed amino acid composition % dry bases.		
Arginine	4.3	2.9
Histidine	1.6	1.4
Isoleucine	2.5	2.1
Leucine	4.1	4.9
Lysine	4.2	2.5
Methionine	1.2	0.7
Phenylalanine	2.5	2.9
Threonine	2.5	1.6
Tyrosine	1.8	1.8
Valine	3.1	2.4
IAA	27.8	23.1
DAA	28	32
IAA/DAA	0.99	0.72

IAA, sum of indispensable amino acids; DAA, sum of dispensable amino acids.

[1]As recommended by NRC (1993).

3.2.3. Growth traits measurement

Growth measurements (weight and length) were recorded before the onset of the experimental diet (at 28 dpf) and after experimental feeding (at 70 and 98 dpf). At 28 dpf, a sample of about 20% of the fish (n ~ 25 fish per family) was used to record initial weight (mg) and length (mm), and these values were used to calculate condition factor ($K= W/L^{-3}/100$) (total n = 607). At 70 dpf (15 fish per family, total n = 720), weight gain (ΔW, mg), length gain (ΔL, mm), condition factor (K) and specific growth rate (SGR) were measured calculated. Weight gain and length gain were calculated as the difference between the two recording stages (ΔW (mg) = weight at 70 dpf – initial weight at 28 dpf; ΔL (mm) = length at 70 dpf – initial length at 28 dpf). At the end of the experimental period, 98 dpf, growth measurements were taken for all fish (total n = 1650). Condition factor was represented as an average at each measured point (28, 70 and 98 dpf). SGR was calculated at 70 and 98 dpf using the equation: 100% x (ln weight2- ln weight1)/t, where t is the number of experimental days. The fish were fasted for 24 h prior to every sampling event. Each fish was weighed (mg) using a scale with a precision of 0.001 g (Acculab VI-3 mg), and length (mm) was measured from mouth to caudal peduncle using digital photography and image analysis software (TPSdig2 v2. 12)[37].

3.2.4. Muscle samples

At 98 dpf, all fish were sacrificed and sexed. Gender was determined by examination of external secondary sexual characteristics, including anal fin and ventral body surface coloration (yellow in males) and body shape (rounded belly in females)[33]. Gender was confirmed by observation of the gonads using a stereoscopic microscope, Nikon model SMZ-10. A muscle sample was stored in an RNAlater ® at -80°C until RNA extraction for gene expression analysis. All animal handling procedures were approved by the Committee of Animal Bioethics at INTA (Instituto de Nutrición y Tecnología de los Alimentos) at the University of Chile.

3.2.5. Selection of individuals for gene expression analysis

To evaluate the effect of PP diet on gene expression, fish showing the mean weight gain in each population were selected, in order to avoid potentially confounding effects from growth differences among individuals in transcriptional analysis. Additionally, the fish were selected from same three families in each

population, in order to homogenize the genetic background between treatments. Criteria for family selection were: 1) Family with a mean weight gain representative of the mean weight gain in each population; 2) Family with an approximately 1:1 male-to-female ratio; and 3) Family with more than six males and females representative of the mean weight gain in each family. Nine males and nine females (3 per family) were selected in each population.

To evaluate the effect of family variation on gene expression, the three families noted above were evaluated separately. We evaluated three families so that the fish would be representative of the average weight gain in each population.

3.2.6. RNA extraction

Total RNA was extracted from the muscle of each individual fish. The muscle tissue was homogenized in Trizol® (Invitrogen) following the manufacturer´s protocol and using a homogenizer (BioSpec Model 9853G XL). Samples were subjected to electrophoresis on 2% agarose gels to confirm the integrity of the 28S and 18S rRNA bands. RNA quality was measured by a spectrophotometer (UV 1601, Shimadzu) through an absorbance ratio of $260nm_{(A_{260})}/280nm_{(A_{280})}$; values between 1.8 and 2.1 were accepted[38]. RNA concentration was determined by absorbance using the equation ($\mu g \cdot mL^{-1}$): A260 x 40 $\mu g \cdot mL^{-1}$ x dilution factor (total volume/RNA volume). All samples were treated with DNAse I (Fermentas®) following the manufacturer´s protocol to remove possible genomic DNA contamination.

3.2.7. First strand cDNA synthesis

cDNA was generated from 2 µg of total RNA using the Maxima^{TM} First Strand cDNA Synthesis Kit for RT-PCR (Fermentas®) following the manufacturer's protocol. In all cases, a reverse transcriptase minus (RT-) negative control was used to test for genomic DNA contamination. The product of the first strand cDNA synthesis was stored at -80°C until the qRT-PCR runs.

3.2.8. Primers design

Published primers were used to quantify *Myod*, *Myogenin*, *Mrf4*, *Mstn1b* and *Igf1a* [39, 40], and new primers for *Igf2a*, *mTOR*, *Pld1a* were designed using AmplifX 1.4.0 (http://amplix.wprogramas.com).Three reference genes, *β-actin*, eukaryotic elongation factor 1α (*Ef1-α*) and ribosomal protein L13α (*Rpl13-α*),

were used[41] (Anexo 2). Primer sequence qualities were evaluated for possible secondary structures using Integrated DNA Technologies (IDT) tools (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer), and similar melting temperatures between 58–60°C were used. The gene transcript sequences were retrieved from the Ensembl databases (http://www.ensembl.org/Danio_rerio), and the exons for each zebrafish gene were identified. The sequences from other fish and from mRNA of zebrafish reference genes were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to confirm the reading frame of each exon. Amplicon lengths used ranges between 50 and 151 base pairs for each gene. The specificity of the primers was checked using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed by conventional PCR from cDNA.

3.2.9. Real time RT-PCR

Real-time PCR was performed with an ABI 7300 Real-Time PCR system using Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas®) following the manufacturer's protocol. PCR reactions were set up using 160 nmol of each primer for *Mstn1b*; 300 nmol for *Mrf4* and *mTOR*; 400 nmol for *Myod*, *Myogenin*, *Igf1a*, *Igf2a*, *Pld1a* and *Ef1α*. *β-actin* and *Rpl13α* were set up using 200 nmol of primer. Two µl of 10-fold diluted cDNA in a reaction volume of 25 µL were used. PCR was run with a 10 min activation and denaturation step at 95°C, followed by 40 cycles at 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Specificity of the reactions was verified using melting curve analysis and absence of primer dimmer. Standard curves were obtained for each cDNA template by plotting Ct values against the $\log_{10}$ of five different dilutions of a cDNA mix solution of all samples analyzed. Real-time PCR efficiency (E) was calculated from a standard curve according to the equation $E = 10^{(-1/slope)}$[42]. All amplifications had a PCR efficiency value of approximately 1.9. The relative expression levels of data from the three potential housekeeping genes in muscle tissue of males and females subjected to the two experimental diets were entered into the GeNorm program to calculate gene expression stability values[43]. Two reference genes, *Ef1α*, and *Rpl13α*, were more stable for males, and *β-actin* and *Rpl13α* were more stable for females. These genes were selected as endogenous control genes in each sex, and zebrafish fed with FM diet were used as calibrators.

3.2.10. Statistical analysis

At 28 dpf, initial weight, length and condition factor were analyzed by one-way analysis of variance (ANOVA) with Student–Newman–Keuls's multiple comparison test (SNK) to compare fries's growth variability among families (significance level $\alpha = 0.05$). At 70 and 98 dpf, the data were analyzed by two-way analysis of variance (ANOVA) with SNK to evaluate fixed effects (diet, family and gender) using the SPSS V8.0 GML procedure (SPSS, 1998)[44]. For gene expression analysis, a Mann–Whitney test was used to determine significant differences between treatments (FM diet vs. PP diet) and among families, using a significance level of .05.

3.3. Results

3.3.1. Growth traits in populations of zebrafish fed with FM and PP diet

Fish had an initial weight, length and condition factor of 1.62 ± 0.03 mg, 5.27 ± 0.03 mm and 1.10 ± 0.02, respectively (n=607). At 70 dpf, fish fed with the FM diet (n=349) had significantly greater ΔW (66.61 ± 1.69 mg vs. 42.11 ± 1.10 mg), ΔL (9.95 ± 0.12 mm vs. 8.66 ± 0.12 mm), K (1.78 ± 0.01 vs. 1.51 ± 0.01) and SGR (8.65 ± 0.07 vs. 7.59 ± 0.07) than fish fed with the PP diet (n=342) ($P<0.01$). At 98 dpf, growth in males fed with the FM diet reached greater ΔW (159.84 ± 2.0 mg vs. 126.32 ± 2.0 mg) than males fed with PP diet ($P<0.01$). Females fed with the FM diet reached greater ΔW (198.71 ± 2.7 mg. vs. 149.88 ± 3.23 mg) than females fed with the PP diet ($P<0.01$). The same trend of higher values for ΔL, K and SGR was observed in fish fed with FM diet compared to PP diet when fish were classified according to sex, as shown in Table 3.2. At 98 dpf, males fed with the FM diet had 27% higher weight gain (+34 mg) than males fed with the PP diet, and females fed with the FM diet had a 33% higher weight gain (+48.8 mg) compared with those fed with PP diet.

Table 3.2. Growth measurements at 98 days post fertilization (dpf) (mean ± SE).

	Males			Females		
	FM diet N=327	PP diet N=440		FM diet N=449	PP diet N=339	
Δ Weight (mg)	159.84 ± 2.0	126.32 ± 2.0	**	198.71 ± 2.7	149.88 ± 3.23	**
Δ Length (mm)	15.04 ± 0.08	14.14 ± 0.10	**	15.48 ± 0.10	14.59 ± 0.10	**
Condition Factor (K)	1.88 ± 0.01	1.68 ± 0.01	**	2.19 ± 0.02	1.85 ± 0.01	**
Specific Growth Rate	6.49 ± 0.03	6.13 ± 0.03		6.86 ± 0.03	6.47 ± 0.03	

** indicates significant differences by treatment (P<0.01).

3.3.2. Variability of growth among 24 experimental families

Family variations in growth are plotted in Figure 3.2. At 28 dpf, variability among families was represented using initial weight (mg), and statistical analysis showed significant differences among families (P<0.01) (Anexo 3). Figure 3.2(A) shows the families ordered by increase in initial weight before initiation of experimental feeding. At 70 dpf, there were significant differences in weight gain by diet and family (P<0.01) (Anexo 4). Figure 3.2(B) shows the families fed with FM diet ordered from lowest to highest weight gain, whereas the families fed with PP diet are shown irrespectively of ranking. At 98 dpf, there were significant differences in weight gain by diet, family and gender (P<0.01) (Anexo 5 and 6). Figure 3.2(C) shows the males from all families fed with FM diet ordered from lowest to highest weight gain, whereas the males from families fed with PP diet are shown irrespectively of ranking. Figure 3.2(D) shows the females from all families fed with FM diet ordered from lowest to highest weight gain, whereas the females from families fed with PP diet are shown irrespectively of ranking. Figure 3.2(B), 3.2(C) and 3.2(D) show that some families tend to maintain their rank from initial weight to 70 and 98 dpf. However, in most families fed with FM and PP diet, initial weight gain ranking is not maintained. Families showed an individual growth response to each diet.

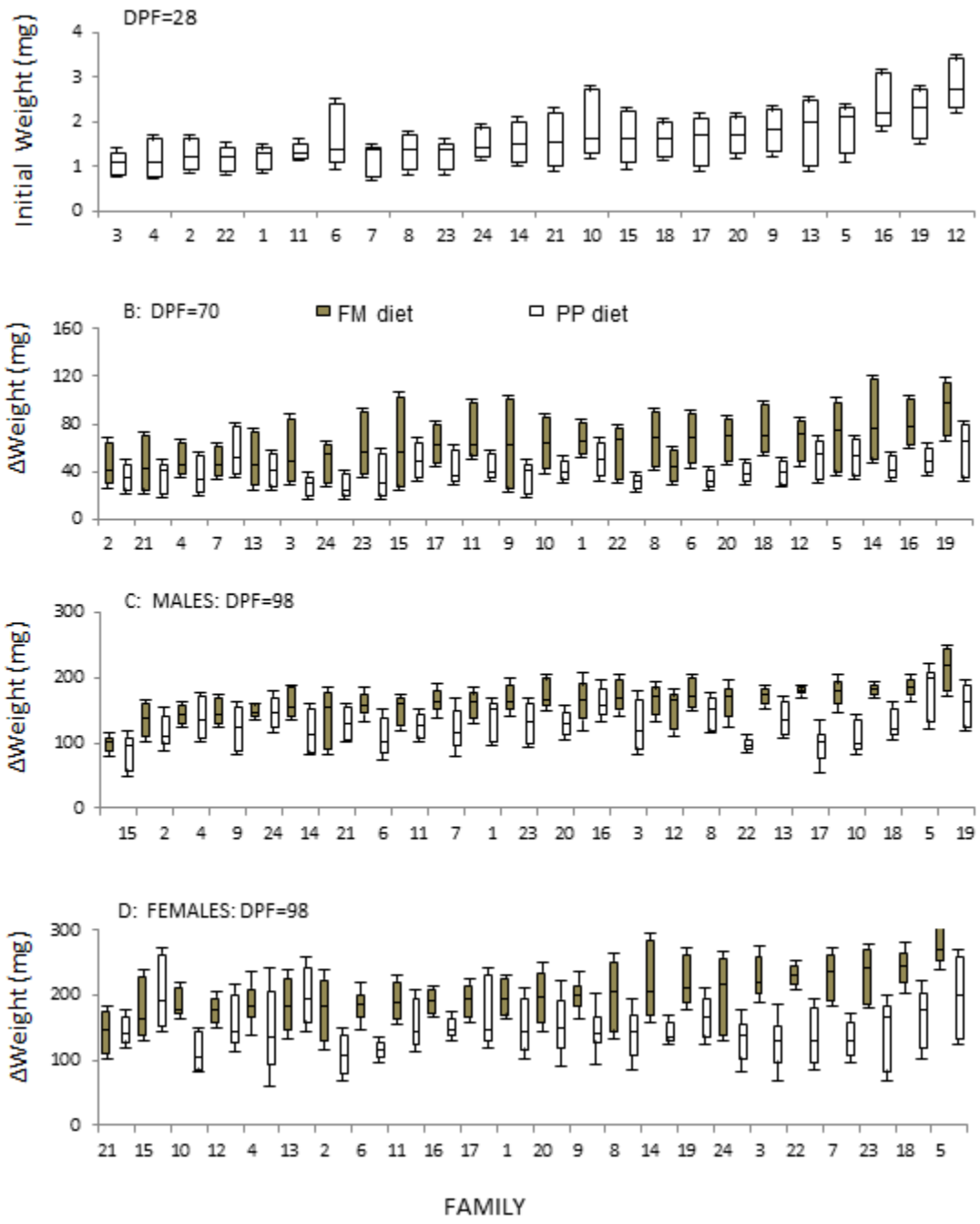

Figure 3.2- Growth variability among 24 families at 28, 70 and 98 dpf. Box and whisker plots are shown (median, 25-75% quartile, maximum and minimum value). A) Initial weight among families before experimental feeding at 28 days post fertilization (dpf) (~25 fish per family). B) Variability in weight gain among families fed with FM and PP diets at 70 dpf (~ 15 fish per family). C) Variability in weight gain of males among families fed with FM and PP diets at 98 dpf. D) Variability in weight gain of females among families fed with FM and PP diets at 98 dpf. Families in B, C and D are shown ordered by increasing mean Δ weight of families fed with FM diet.

3.3.3. Selection of families for gene expression analysis

Considering that at 98 dpf there were large differences in weight gain among families in males and females (see Figures 3.2C and 3.2D), the fish were selected from three same families (4, 7 and 23) with the mean weight gain in each population (see Materials and Method, Point 2.5). Table 3.3 shows the mean weight gain in each family selected for gene expression analysis as well as the values in males and females selected from these families (mean± SE).

Table 3.3. Families selected for gene expression analysis and weight gain (ΔW, mg) in males and females at 98 dpf (mean ± SE).

Diets	Family selection	ΔW family	ΔW males	ΔW females
FM	4	164.10 ± 6.68 (n=39)	140.14 ± 7.40 (n=18)	184.64 ± 11.45 (n=21)
	7	202.25 ± 11.06 (n=25)	161.86 ± 9.93 (n=10)	229.18 ± 13.54 (n=15)
	23	191.64 ± 10.42 (n=32)	164.16 ± 7.40 (n=18)	226.96 ± 14.02 (n=14)
PP	4	142.53 ± 8.74 (n=36)	137.10 ± 8.36 (n=21)	150.13 ± 14.55 (n=15)
	7	128.20 ± 7.75 (n=27)	120.76 ± 9.57 (n=16)	139.03 ± 16.99 (n=11)
	23	139.01 ± 9.53 (n=27)	133.48 ± 9.57 (n=16)	147.05 ± 16.99 (n=11)

3.3.4. Gene expression in muscle of zebrafish fed FM and PP diets.

In order to evaluate the effects of PP diet on gene expression, data analysis was performed for all three selected families separately by sex. Males fed with the PP diet showed a significant over-expression ($P<0.05$) in *Myogenin* (X5.0 ± 0.67) and *Mrf4* (X3.42 ± 0.56) as compared with gene expression of males fed with the FM diet, whereas *Igf2*a was under-expressed (X0.71 ± 0.09) (Figure 3.3A). The other genes studied (*Igf1a*, *Mstn1b*, *mTOR*, *Pld1a*, *Myod*) did not show significant differences in gene expression by diet in males (Figure 3.3A). In females, there were no statistical differences in gene expression by diet for any of the eight genes studied (Figure 3.3B).

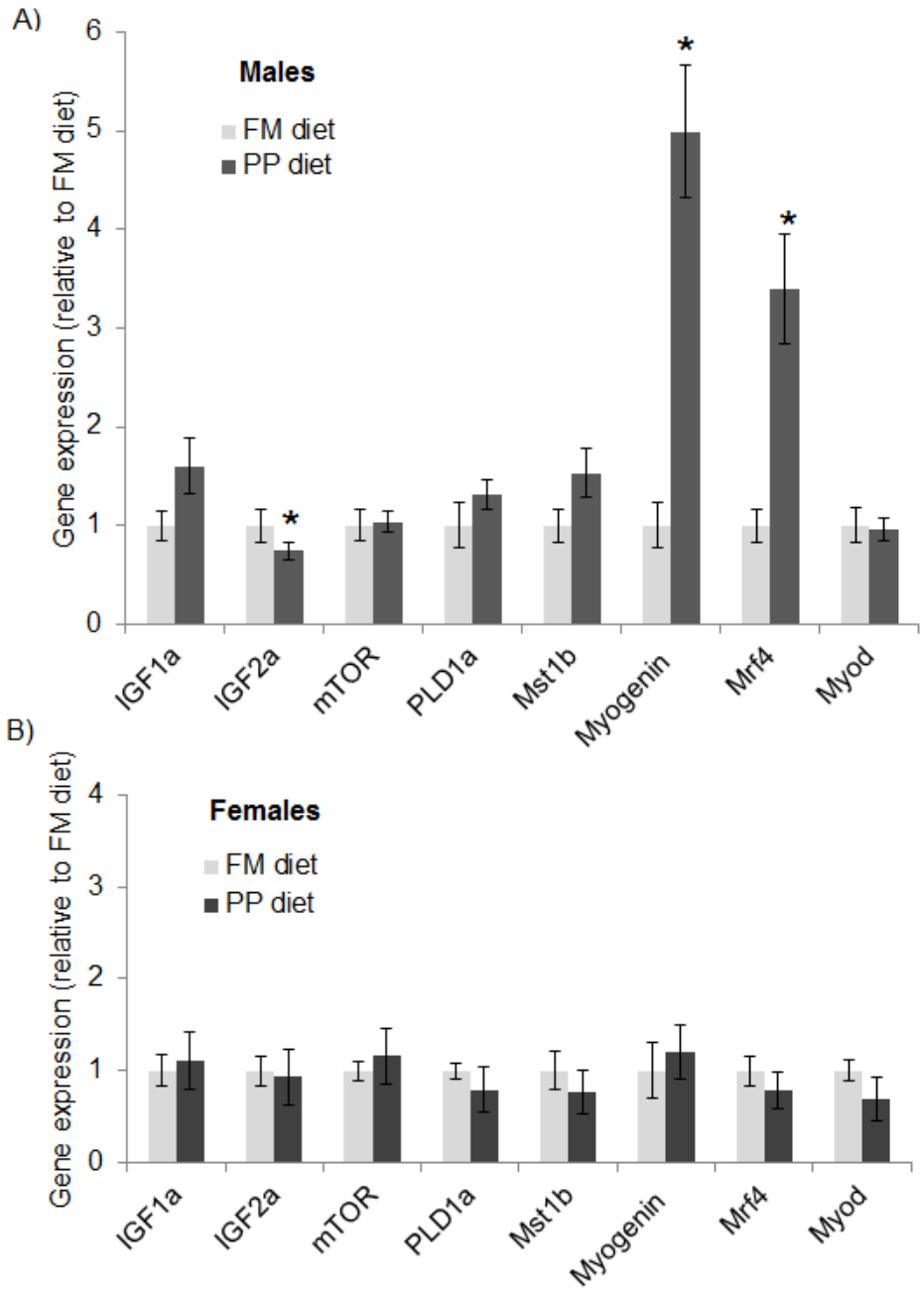

Figure 3.3. Gene expression in muscle of males and females fed with FM and PP diets. A) Real-time PCR quantification of *Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mstn1b*, *Myogenin*, *Mrf4* and *Myod* in muscle of males. Bars indicate the mean and standard error of 27 data points (9 biological samples in each treatment x 3 replicates). Significant differences indicated with (*) were determined using Mann-Whitney test ($P<0.05$). B) Real-time PCR quantification of the same genes in the muscle of females. No significant differences were detected after Mann-Whitney test.

In order to evaluate the effects of family variation on gene expression, data were analyzed in each family separately. In males, mRNA levels of *Myogenin* and *Mrf4* were significantly higher in all three selected families (number 4, 7 and 23) fed with PP diet as compared with FM diet (Figure 3.4A, 3.4B). *Igf2a* expression was decreased in all families fed with PP diet, but only one family (23) showed a significant difference by diet (Figure 3.4C). *Igf1a* and *Pld1a* expression was significantly increased in family 4 in response to PP diet (Figure 3.4D, 3.4E), and *Mstn1b* was significantly increased in families 4 and 23 (Figure 3.4F). In females, *Igf1a*, *Myogenin*, *mTOR* and *Myod* expression was significantly increased in family 23 in response to PP diet (Figure 3.5A, 3.5B, 3.5C, 3.5D) whereas *Pld1a* expression showed was significantly decreased in family 4 in response to PP diet (Figure 3.5E).

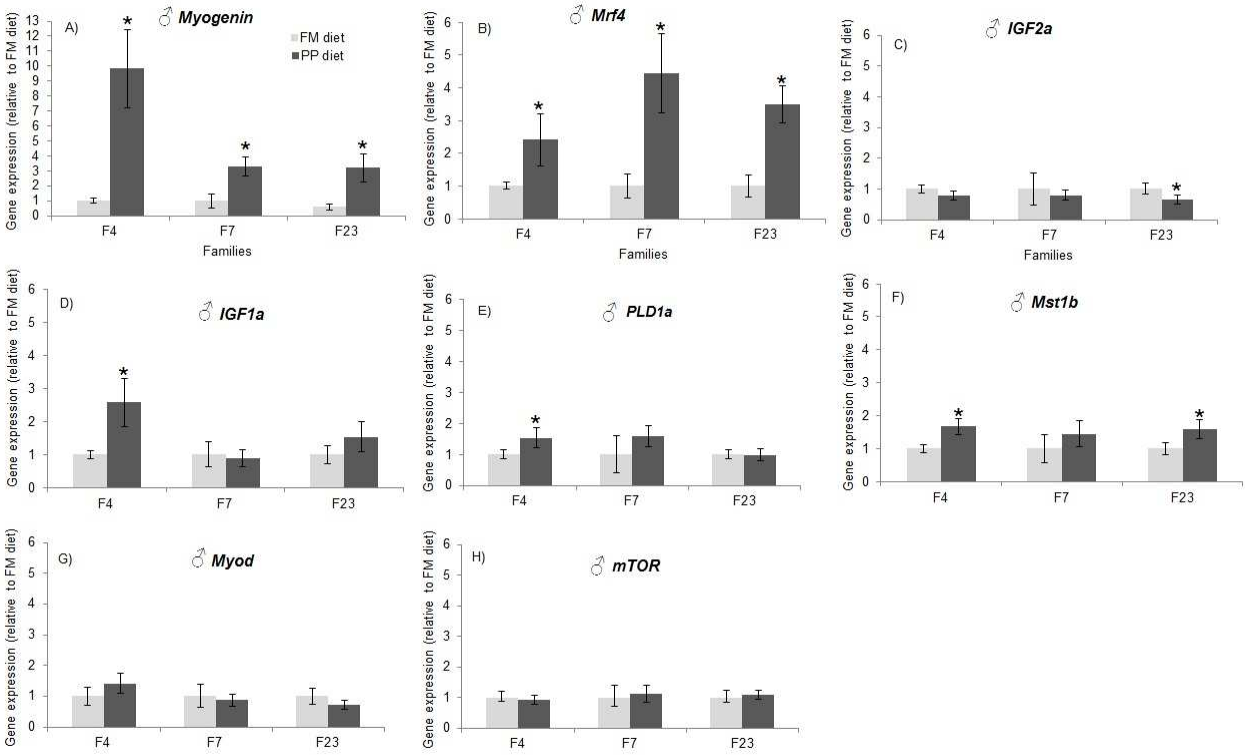

Figure 3.4. Gene expression in muscle of males among three families. Real-time PCR quantification of *Myogenin*, *Mrf4*, *Myod*, *Igf2a*, *Igf1a*, *Pld1a*, *Mstn1b*, *Myod* and *mTOR* in muscle of males. Bars indicate the mean and standard error of 9 data points (3 biological samples per family x 3 replicate). Significant differences indicated with (*) were determined using Mann-Whitney test ($P<0.05$). A-B) Myogenin and *Mrf4* showed a significant increase in three families (number 4, 7 and 23) fed with PP diet as compared to FM diet. C) *Igf2a* showed significant decrease in family number 23. D-E) *Igf1a* and *Pld1a* showed a significant increase in family number 4. F) Mstn1b also showed a significant increase in families number 4 and 23. G-H) *Myod* and *mTOR* did not show statistical differences in the three families fed with PP diet as compared to FM diet.

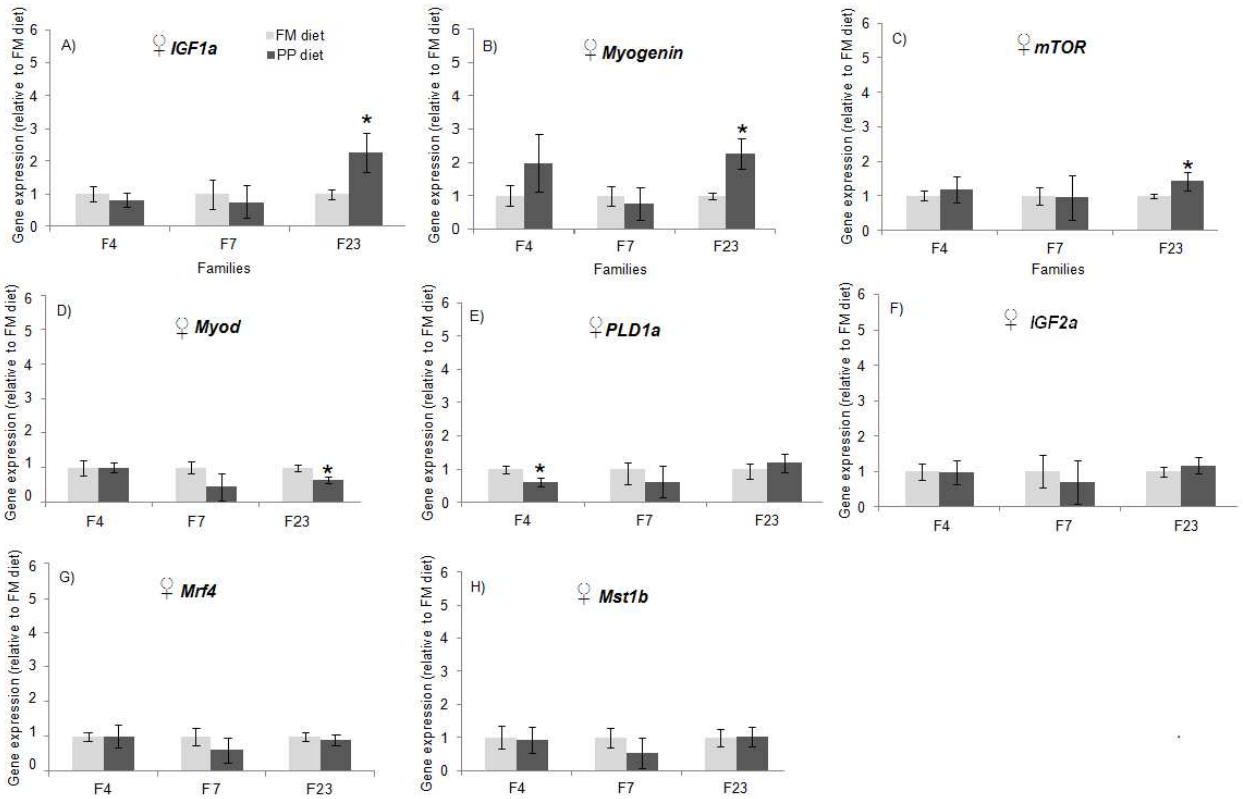

Figure 3.5. Gene expression in muscle of females among three families. Real-time PCR quantification of *Igf1a*, *Myogenin*, *mTOR*, *Myod*, *Pld1a*, *Igf2a*, *Mrf4* and *Mstn1b* in muscle of females. Bars indicate the mean and standard error of 9 data points (3 biological samples per family x 3 replicate). Significant differences indicated with (*) were determined using Mann-Whitney test ($P<0.05$). A-D) *Igf1a*, *Myogenin*, *mTOR* and *Myod* showed significant increases in family number 23 in response to PP diet. E) Pld1a showed significant decreases in family number 4. *Igf2a*, *Mrf4* and *Mstn1b* did not show statistical differences in the three families fed with PP diet as compared to FM diet.

3.4. Discussion

3.4.1. Variation in zebrafish growth

This study demonstrated that a PP diet reduces zebrafish growth as compared with a FM diet. At 70 and 98 dpf, growth measurements (weight gain, length gain, condition factor and SGR) were significantly higher for fish fed with FM compared to those fed with PP. These results could be compared with findings in other studies in zebrafish fed with different formulated diets. For example, Robison et al.[33] demonstrated that manipulation of dietary carbohydrate levels (0, 15, 25 and 35%) had a significant effect on body weight and condition factor. In another study, zebrafish were fed with different sources of plant protein

(soy bean + corn genetically modified (GM) and non-GM). Fish fed with GM corn exhibited significantly better growth as compared with fish fed with non-GM corn[45]. Our results can also be compared with studies in farmed fish fed with different percentages PP replacement of FM. Our results are consistent with findings in common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and rainbow trout, which showed a marked reduction in growth when 6–19 g alevins were fed with a diet of 75–100% FM replacement[6, 46-49]. In this study, growth traits were evaluated in zebrafish from alevin to adult stages (28 to 98 dpf) in populations with similar genetic backgrounds. Therefore our results may be attributed principally to differences between diets. The decreased growth in zebrafish fed with PP diet could be related to antinutritional factors in the plant protein diet (wheat, corn and soy concentrate) as well as limited availability of essential amino acids, such as arginine (2.9%), lysine (2.5%) and methionine (0.7). On the other hand, discordant results have also been reported in70–83 g juvenile rainbow trout, which showed similar growth in fish fed with high replacement of FM (66-100%) with soy protein concentrate[10, 11]. These differences between studies may be attributed to the biological developmental stage at the beginning of the experimental period.

Differences at 28 dpf in initial weight among offspring of different families were likely the product of inter-family genetic differences within the population. However, at 70 and 98 dpf, family differences in weight gain varied by diet (see Figures 2B, C and D). These plots show clearly that families with the greatest increases in weight gain fed when with FM diet did not maintain the same ranking when fed with PP diet. This indicates that the genetic components underlying growth phenotype in each family produced a different physiological response depending on the plant or fishmeal protein-based diet.

3.4.2. Plant protein-based diet modulates the expression of *Igf2a, Myogenin* and *Mrf4* in the muscle of zebrafish males.

These results show that *Igf2a*, *Myogenin* and *Mrf4* were regulated by plant nutrients in the muscle of zebrafish males. The somatotropic pathways, including genes such as those for endocrine growth hormone and insulin-like growth factor 1 and 2 (*Igf1* and *Igf2*), positively influence fish growth and are known to play a key role in the regulation of metabolism and physiological processes[22]. A 0.71-fold decrease in *Igf2a* mRNA levels in the muscle of males fed with PP diet as compared with FM diet was shown (Figure 3A). This Igf2a expression pattern could be explained by lower lysine levels in PP diet as compared with FM diet (2.5 vs. 4.2 respectively), as has been shown in Atlantic salmon when fish fed with low lysine (2.9 g/16 g N) showed a 2.6-fold decrease in *Igf2* mRNA levels compared to those fed with medium lysine

levels (4.9 g/16 g N)[50]. *Myogenin* (X5.0 ± 0.67) and *Mrf4* (X3.42 ± 0.56) were over-expressed in fish fed with a PP diet as compared with FM diet (Figure 3A). *Myogenin and Mrf4* are essential factors involved in the late steps of myogenesis and in hypertrophy (increase in size of existing muscle fibers) and hyperplasia (recruitment of new muscle fibers)[51]. They are involved in the differentiation process, generating myotubes which can fuse to form short myoblast-myotubes, and fusion events, producing fiber maturation and hypertrophy[23]. The nutritional regulation of muscle is poorly documented in fish. A single report in rainbow trout showed that the substitution of FM with graded levels of PP (50%, 75%, 100% replacement) in diets did not modify the expression of *Myogenin* in fish muscle[6]. This difference in gene expression in response to PP diet between species could be due to different processes involved in muscle growth (hypertrophy in zebrafish and hyperplasia and hypertrophy in rainbow trout)[21, 52].

The other genes studied *Myod*, *Igf1a*, *mTOR* and *Pld1a* did not show significant differences in gene expression by diet in zebrafish (Figure 3A). In relation to the last two genes, there is evidence in the mouse that *mTOR* is regulated by *Pld1* through a nutrient-sensing pathway including the leucine amino acid[25, 26]. In our study the similar leucine level in both diets (4.1% in the FM diet vs. 4.9% the PP diet) could explain the absence of significant difference in *Pld1* mRNA levels, suggesting that *mTOR* expression was not modified. However, the potential role of *Pld1* as an upstream regulator of *mTOR* has not been examined in fish. In females, there were no significant differences in mRNA level expression in any of the eight genes studied (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mstn1b*, *Myogenin*, *Mrf4*, *Myod*) by diet (Figure 3B). The presence of sexual dimorphism in gene expression has been also described in zebrafish liver and brain[33, 53]. However, our results show that even when all eight genes were expressed in the muscle of males and females, differential expression of *Myogenin*, *Mrf4* and *Igf2a* in response to PP diet was remarkable only in males. That would suggest that males were more affected by PP diets than females.

In order to evaluate the familiar effect on gene expression, the data were analyzed for each family separately. The familiar variation effect was observed in males and females among families. In males, *Myogenin* and *Mrf4* were over-expressed in response to PP diet in all three families selected (Figure 4A, 4B). This suggests that the expression of these genes in males was modulated principally by the plant ingredients rather than genetic differences among families. *Igf2a*, *Igf1a*, *Pld1a* and *Mstn1b* were differentially expressed in the families 4 and 23 (Figure 4C, 4D, 4E, 4F). In females, *Igf1a*, *Myogenin*, *Myod*, *mTOR* and *Pld1a* were expressed differentially by diet in the same families (4 and 23) (Figure 5A, 5B, 5C, 5D, 5E). These results suggest that differences in gene expression may be influenced not only by dietary components but also by genetic differences that underlie each family. Therefore it is important to

pay attention to both sources of variation in explaining the modulation of gene expression in nutrigenomics studies. These results could confirm that the individuals from the families 4 and 23 were more affected by PP diet even when their growth was not modified.

A recent study evaluated the effect of genotype on liver transcriptome in Atlantic salmon fed with a vegetable oil-based diet. Diet modulated the expression of genes regulating metabolism, especially those for lipid and carbohydrate, as well as immune response[19]. Genetic differences among families modulated signaling pathways and had a lower impact on metabolism-related genes[19]. Our study in zebrafish showed that a plant protein diet and genetic components underlying family variation produced separate effects on the modulation of gene expression related to growth in male and female zebrafish. This approach opens the door to evaluating the whole transcriptome and identifying metabolic pathways affected by plant nutrients in zebrafish, which could be tested in farmed fish using comparative genomics.

Acknowledgements

The first author received a doctoral fellowship from the Consorcio Empresarial de Genética y Desarrollo Biotecnológico para la Industria Salmonera (Aquainnovo S.A). This work was supported by a fellowship of Apoyo a la Realización de Tesis Doctoral, from Conicyt AT-24091052 and Aquainnovo S.A. PEU wishes to thank the research group of the Laboratory in Genetic and Biotechnology in Aquaculture at the Universidad de Chile where the fish were reared and the Research Group of the Animal Genomic Laboratory at the University of California Davis, USA.

3.5. References

1. FAO: El estado mundial de la pesca y la acuicultura. Organización de las Naciones Unidas para la Alimentación y la Agricultura. In: Italy R, (ed), 2012.

2. Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, et al.: Aquaculture: global status and trends. Philos Trans R Soc Lond B Biol Sci. 2010;365:2897-912.

3. Bórquez AS, Hernández AJ: Status and trends on the use of small pelagic fish species for reduction fisheries and for consumption in Chile In: Hasan MR and Halwart M,(eds). Fish as feed inputs for aquaculture: practice, sustainability and implications FAO Fisheries and Aquaculture Technical Paper, Rome, 2009.

4. Hardy RW: Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research. 2010;41:770-6.

5. Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, et al.: Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences. 2009;106:15103-10.

6. Alami-Durante H, Médale F, Cluzeaud M, Kaushik SJ: Skeletal muscle growth dynamics and expression of related genes in white and red muscles of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture. 2010;303:50-8.

7. Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF, et al.: Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (Sparus aurata). Aquaculture. 2004;232:493-510.

8. Krogdahl Å, Penn M, Thorsen J, Refstie S, Bakke AM: Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquaculture Research. 2010;41:333-44.

9. Mundheim H, Aksnes A, Hope B: Growth, feed efficiency and digestibility in salmon (Salmo salar L.) fed different dietary proportions of vegetable protein sources in combination with two fish meal qualities. Aquaculture. 2004;237:315-31.

10. Kaushik SJ, Cravedi JP, Lalles JP, Sumpter J, Fauconneau B, Laroche M: Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, Oncorhynchus mykiss. Aquaculture. 1995;133:257-74.

11. Olli JJ, Krogdahi Å: Nutritive Value of Four Soybean Products as Protein Sources in Diets for Rainbow Trout (Oncorhynchus mykiss, Walbaum) Reared in Fresh Water. Acta Agriculturae Scandinavica, Section A - Animal Science. 1994;44:185 - 92.

12. Mutch DM, Wahli W, Williamson G: Nutrigenomics and nutrigenetics: the emerging faces of nutrition. The FASEB Journal. 2005;19:1602-16.

13. Benedito-Palos L, Saera-Vila A, Calduch-Giner J-A, Kaushik S, Pérez-Sánchez J: Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (Sparus aurata L.): Networking of systemic and local components of GH/IGF axis. Aquaculture. 2007;267:199-212.

14. FrØYstad MK, Lilleeng E, Bakke-Mckellep AM, Vekterud K, Hemre GI, Krogdahl Å: Gene expression in distal intestine of Atlantic salmon (Salmo salar L.) fed genetically modified soybean meal. Aquaculture Nutrition. 2008;14:204-14.

15. FrØYstad-Saugen MK, Lilleeng E, Bakke-McKellep AM, Vekterud K, Valen EC, Hemre GI, et al.: Distal intestinal gene expression in Atlantic salmon (Salmo salar L.) fed genetically modified maize. Aquaculture Nutrition. 2009;15:104-15.

16. Lilleeng E, Froystad MK, Ostby GC, Valen EC, Krogdahl A: Effects of diets containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (Salmo salar L). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology. 2007;147:25-36.

17. Panserat S, Kolditz C, Richard N, Plagnes-Juan E, Piumi F, Esquerré D, et al.: Hepatic gene expression profiles in juvenile rainbow trout (Oncorhynchus mykiss) fed fishmeal or fish oil-free diets. British Journal of Nutrition. 2008;100:953-67.

18. Roher J, Diaz M, Mackenzie S, Krasnov A, Planas J: Aproximaciones genómicas al crecimiento y nutricion de peces In: Genética y Genómica en Acuicultura Monteros JEdl, (ed), pp. 890, Madrid, 2007.

19. Morais S, Pratoomyot J, Taggart J, Bron J, Guy D, Bell J, et al.: Genotype-specific responses in Atlantic salmon (Salmo salar) subject to dietary. BMC Genomics. 2011;12:255.

20. De-Santis C, Jerry DR: Candidate growth genes in finfish — Where should we be looking? Aquaculture. 2007;272:22-38.

21. Johnston I, Macqueen D, Watabe S: Molecular biotechnology of development and growth in fish muscle. In: Tsukamoto K, Kawamura T, Takeuchi T, Douglas Beard T, Kaiser M (eds). Fisheries for global welfare and environment 5th World Fisheries Congress, Scotland, 2008.

22. Moriyama S, Ayson FG, Kawauchi H: Growth regulation by insulin-like growth factor-I in fish. Biosci Biotechnol Biochem. 2000;64:1553-62.

23. Johnston I: Environment and plasticity of myogenesis in teleost fish. J Exp Biol 2006 Jun;209(Pt 12):2249-64. 2006;209:2249-64.

24. Clemmons DR: Role of IGF-I in skeletal muscle mass maintenance. Trends in Endocrinology & Metabolism. 2009;20:349-56.

25. Kimball SR, Jefferson LS: New functions for amino acids: effects on gene transcription and translation. The American Journal of Clinical Nutrition. 2006;83:500S-7S.

26. Yoon M-S, Chen J: PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. Journal of Cell Science. 2008;121:282-9.

27. Atchley WR, Fitch WM, Bronner-Fraser M: Molecular evolution of the MyoD family of transcription factors. Proceedings of the National Academy of Sciences. 1994;91:11522-6.

28. Hinits Y, Osborn DPS, Carvajal JJ, Rigby PWJ, Hughes SM: Mrf4 (myf6) is dynamically expressed in differentiated zebrafish skeletal muscle. Gene Expression Patterns. 2007;7:738-45.

29. Acosta J, Carpio Y, Borroto I, González O, Estrada MP: Myostatin gene silenced by RNAi show a zebrafish giant phenotype. Journal of Biotechnology. 2005;119:324-31.

30. McPherron AC, Lawler AM, Lee S-J: Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. Nature. 1997;387:83-90.

31. Ulloa PE, Iturra P, Neira R, Araneda C: Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. Reviews in Fish Biology and Fisheries. 2011:1-18.

32. Brand M, Granato M, Nüsslein-Volhard C: Keeping and raising zebrafish. In: Zebrafish Nüsslein-Volhard C, Dahm R, (eds), pp. 7-37. Oxford University Press, Oxford, 2002.

33. Robison BD, Drew RE, Murdoch GK, Powell M, Rodnick KJ, Settles M, et al.: Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (Danio rerio). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2008;3:141-54.

34. Meinelt BT, Schulz C, Wirth M, Kürzinger H, Steinberg C: Dietary fatty acid composition influences the fertilization rate of zebrafish(Danio rerio Hamilton-Buchanan). Journal of Applied Ichthyology. 1999;15:19-23.

35. Meinelt T, Schulz C, Wirth M, Kurzinger H, Steinberg C: Correlation of diets high in n-6 polyunsaturated fatty acids with high growth rate in zebrafish (Danio rerio). Comp Med. 2000;50:43-5.

36. Bidlingmeyer, A. B, Cohen, A. S, Tarvin, L. T, et al.: A new, rapid, high-sensitivity analysis of amino acids in food type samples. Washington, : Association of Official Analytical Chemists; 1987.

37. Adams DC, Rohlf FJ, Slice DE: Geometric morphometrics: Ten years of progress following the 'revolution'. Italian Journal of Zoology. 2004;71:5 - 16.

38. Nolan T, Hands RE, Bustin SA: Quantification of mRNA using real-time RT-PCR. Nat Protocols. 2006;1:1559-82.

39. Hu M-C, Gong H-Y, Lin G-H, Hu S-Y, Chen MH-C, Huang S-J, et al.: XBP-1, a key regulator of unfolded protein response, activates transcription of IGF1 and Akt phosphorylation in zebrafish embryonic cell line. Biochemical and Biophysical Research Communications. 2007;359:778-83.

40. Lee C-Y, Hu S-Y, Gong H-Y, Chen MH-C, Lu J-K, Wu J-L: Suppression of myostatin with vector-based RNA interference causes a double-muscle effect in transgenic zebrafish. Biochemical and Biophysical Research Communications. 2009;387:766-71.

41. Tang R, Dodd A, Lai D, McNabb WC, Love DR: Validation of Zebrafish (Danio rerio) Reference Genes for Quantitative Real-time RT-PCR Normalization. Acta Biochimica et Biophysica Sinica. 2007;39:384-90.

42. Pfaffl MW: A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research. 2001;29:e45.

43. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al.: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:RESEARCH0034.

44. Zar JH: Biostatistical analysis: Prentice Hall; 2010.

45. Sissener NH, Johannessen LE, Hevrøy EM, Wiik-Nielsen CR, Berdal KG, Nordgreen A, et al.: Zebrafish (Danio rerio) as a model for investigating the safety of GM feed ingredients (soya and maize);

performance, stress response and uptake of dietary DNA sequences. British Journal of Nutrition. 2010;103:3-15.

46. Fontaínhas-Fernandes A, Gomes E, Reis-Henriques MA, Coimbra J: Replacement of Fish Meal by Plant Proteins in the Diet of Nile Tilapia: Digestibility and Growth Performance. Aquaculture International. 1999;7:57-67.

47. Médale F, Boujard T, Vallée F, Blanc D, Mambrini M, Roem A, et al.: Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (Oncorhynchus mykiss) fed increasing dietary levels of soy protein concentrate. Aquatic Living Resources. 11:239-46.

48. Pongmaneerat J, Watanabe T, Takeuchi T, Satoh T: Use of different protein meals as partial or total substitution for fish meal in carp diets. Tokyo, JAPON: Nippon Suisan Gakkai; 1993.

49. Vilhelmsson OT, Martin SAM, Médale F, Kaushik SJ, Houlihan DF: Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (Oncorhynchus mykiss). British Journal of Nutrition. 2004;92:71-80.

50. Hevrøy EM, El-Mowafi A, Taylor RG, Olsvik PA, Norberg B, Espe M: Lysine intake affects gene expression of anabolic hormones in atlantic salmon, Salmo salar. General and Comparative Endocrinology. 2007;152:39-46.

51. Johnston IA, Bower NI, Macqueen DJ: Growth and the regulation of myotomal muscle mass in teleost fish Journal of Experimental Biology 2011;214 1617-28

52. Du SJ: Molecular regulation of fish muscle development and growth. In: Korzh V, Gong Z, (eds). Molecular Aspects in Fish and Marine Biology, Singapure, 2004.

53. Santos E, Kille P, Workman V, Paull G, Tyler Ch: Sexually dimorphic gene expression in the brains of mature zebrafish. Comp Biochem Physiol A Mol Integr Physiol 2008;149:314-24.

Chapter 4. Candidate gene expression in muscle of adult zebrafish of lower and higher growth in response to plant protein-based diet

Manuscript in preparation

Candidate gene expression in muscle of adult zebrafish of lower and higher growth in response to plant protein-based diet

Pilar E. Ulloa[1,3], Andrea A. Peña[2], Carla D. Lizama[3], Cristian Araneda[3], Patricia Iturra[4], Roberto Neira[3,5]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Av. Francisco Salazar 01145, Casilla 54-D, Temuco, Chile.

[2] Laboratorio de Genética e Inmunología Molecular. Pontificia Universidad Católica de Valparaíso. Av. Brasil 2950, Valparaíso, Chile.

[3]Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile.

[4]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70061-7, Santiago, Chile.

[5]AQUAINNOVO S.A., Polpaico 037, Puerto Montt, Chile.

Abstract

The focus of this study was to examine the gene expression related to growth in muscle of lower and higher growth zebrafish (*Danio rerio*) fed with a plant protein-based diet. A zebrafish population of 24 families was fed with a balanced plant protein diet from 35 dpf (days post fertilization) to 98 dpf. For gene expression analysis the individuals were selected according to growth phenotype at 98 dpf. Measurements were taken to evaluate growth response to a diet. From 440 males and 339 females, five percent (n=17) in both ends of the normal distribution for weight gain were selected in each case. From this individuals eight lower growth fish (average weight = 52 mg in males and 58 mg in females) and eight higher growth fish (average weight = 228 mg in males and 220 mg in females) were selected to gene expression analysis. Eight genes (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Myostatin1b*) were evaluated in muscle of males and females. In males with higher growth, *Myogenin* was under-expressed (X0.31 ± 0.08) as compared to lower growth, whereas *Pld1a* was over-expressed (X1.31 ± 0.08). In females with higher growth, *Myostatin1b* (X1.82 ± 0.31) and *mTOR* (X1.55 ± 0.27) were over-expressed as compared to lower growth. This study identified those genes involved in growth, which are modulated by genetic background that underlies the phenotype in response to plant nutrients in males and females.

4.1. Introduction

The limited availability of fishmeal associated to high fish production has led to the exploration of alternative plant protein sources in the diet of fish (FAO, 2012). The most common plant protein incorporated to fish diet has been soy flour. However, the uses of corn, peas, lupins, canola, barley, and wheat have been gradually increasing in the diet formulation(Naylor et al., 2009). Diets for herbivorous and carnivorous fish normally contain between 15% and 45% of soybean meal, however is anticipated that over the next 10 to 12 years, the aquaculture growth activity will depend strongly on plant protein sources (FAO, 2012). Animal and plant protein sources differ in the amino acid profile, relative amounts of protein, metabolizable energy, and mineral contents (Krogdahl et al., 2010) These differences between protein sources alter the nutritional fish states, which are reflected in changes in phenotype such as growth (Alami-Durante et al., 2010; Gómez-Requeni et al., 2004; Mundheim et al., 2004; Krogdahl et al., 2003). Growth is a quantitative trait that varies among individuals in a population and the phenotypic variability depends on the individual genotype and environmental factors (Falconer and Mackay, 1996). Because there is a change in diet, we are interested to study physiological and molecular responses underlying the growth phenotype. The expression of candidate gene related to growth using a physiological model as comparison between adult fish of lower and higher growth in response to a plant protein diet has not been documented in fish. We hypothesized that changes in gene expression in the fish muscle between individuals with different phenotype, could give account of genetic background. This information could be relevant, firstly to implement new strategies that reveal the most efficient fish to utilize plant protein nutrient in the diet and secondly to identify those genes related to growth, which are modulated by genetic background that underlies the phenotype in response to diet. The main objective of this study was to evaluate the expression of eight candidate genes related to growth (*Igf1a, Igf2a, mTOR, Pld1a, Mrf4, Myod, Myogenin* and *Myostatin1b*) in muscle of adult zebrafish with lower and higher growth in response to a plant protein-based diet.

4.2. Material and Methods

4.2.1. Rearing fish

A zebrafish population of 24 experimental families (single pair mating from no related wild-type strain) was generated in order to examine growth response with a wide genetic variability population. Larvae were reared under the best physical and chemical parameters of water ($25 \leq T$ °C ≤ 28.5, $7 \leq pH \leq 8$; hardness > 100 mg $CaCO3 \bullet L^{-1}$ and with a photoperiod of 14 h light:10 h dark (Brand et al., 2002). From 35 to 98 dpf, the fish were fed with a diet that contained plant protein as a unique protein source (wheat, corn and soy concentrate) in their diet. The Table 4.1 shows the ingredients formulation and composition in the experimental diet. The diet was supplemented with Lysine amino acids to match indispensable amino acid requirements of cyprinids fish (like zebrafish) (NRC, 1993).

Table 4.1. Ingredients formulation and analytical composition in the experimental diet.

	Experimental diet
Ingredients ($g \cdot Kg^{-1}$):	
Fishmeal	0
Corn gluten meal	0.150
Wheat gluten	0.350
Soy protein concentrate	0.270
Raw starch	0.075
Fish oil	0.085
Vitamin/mineral premix[1]	0.040
Choline chloride	0.005
Lysine	0.005
Ca(H2PO4)2	0.020
Total	1
Analytical composition (dry bases)	
Dry matter (%)	95.2
Digestible protein (%)	57.8
Digestible lipids (%)	7.10
Starch (%)	22.5
Ash (%)	6.2
Gross energy ($cal \cdot kg^{-1}$)	391

4.2.2. Growth trait measurement

Growth measurements (weight and length) were recorded before the onset of the experimental diet (at 28 dpf) and after experimental feeding (at 70 and 98 dpf), and these values were used to calculate condition factor ($K= W/L^{-3}/100$) in each point measured. At 98 dpf the weight gain (mg) curve was constructed from data collected at 28, 70 and 98 dpf. Each fish was weighed (mg) using a scale with a precision of 0.001 g (Acculab VI-3 mg) and length (mm) was measured from the mouth to the caudal peduncle using digital photography and image analysis software (TPSdig2 v2. 12) (Adams et al., 2004).

4.2.3. Muscle samples

At 98 dpf all the fish were sacrificed and sexed. A sample of muscle was extracted and stored in ~~an~~ RNAlater ® at -80°C until the RNA extraction for gene expression analysis. All procedures for animal handling were approved by the Committee of Animal Bioethics at INTA (Instituto de Nutrición y Tecnología de los Alimentos) at the University of Chile.

4.2.4. Selection of individuals for gene expression analysis

For gene expression analysis the fish were selected according to final growth (weight gain, mg) at 98 dpf. In order to avoid potential confound effects in transcriptome analysis between gender, males (n=440) and females (n=339) were analyzed separately. From these fish, five percent in both ends of the normal distribution for weight gain were selected in each case (n ~17 fish per extreme). From these individuals, eight fish with lower growth (mean weight gain: 52 mg ♂ and 63 mg ♀) and eight fish with higher growth (means weight gain: 228 mg ♂and 294 mg ♀) were selected. These individuals represented to different families within the fish population.

4.2.5. Gene expression procedures

The procedures for gene expression as RNA extraction, First strand cDNA synthesis; Primers design, and real time PCR, were performed as was mentioned in the previous study (Chapter 3).

4.2.6. Statistical analysis

Relative gene expressions for target genes were calculated using the geometric mean of the two most stable reference genes for males (*Ef1α*, and *Rpl13α)* and females (*β-actin* and *Rpl13α)* as normalization factors. The fish with lower growth were used as calibrator sample. A Mann–Whitney test was used to determine significant differences in gene expression between higher and lower growth fish using an α level of .05. Statistical analysis was performed using the SPSS 13.0 statistical package.

4.3. Results

4.3.1. Gene expression in muscle of adult zebrafish of lower and higher growth zebrafish males fed with plant protein-based diet

Males with higher growth response to PP diet showed that *Pld11a* was over-expressed (X1.31 ± 0.08) compared to lower growth males, whereas *Myogenin* was under-expressed (X0.31 ± 0.08) ($P<0.05$) (Figure 4.1).

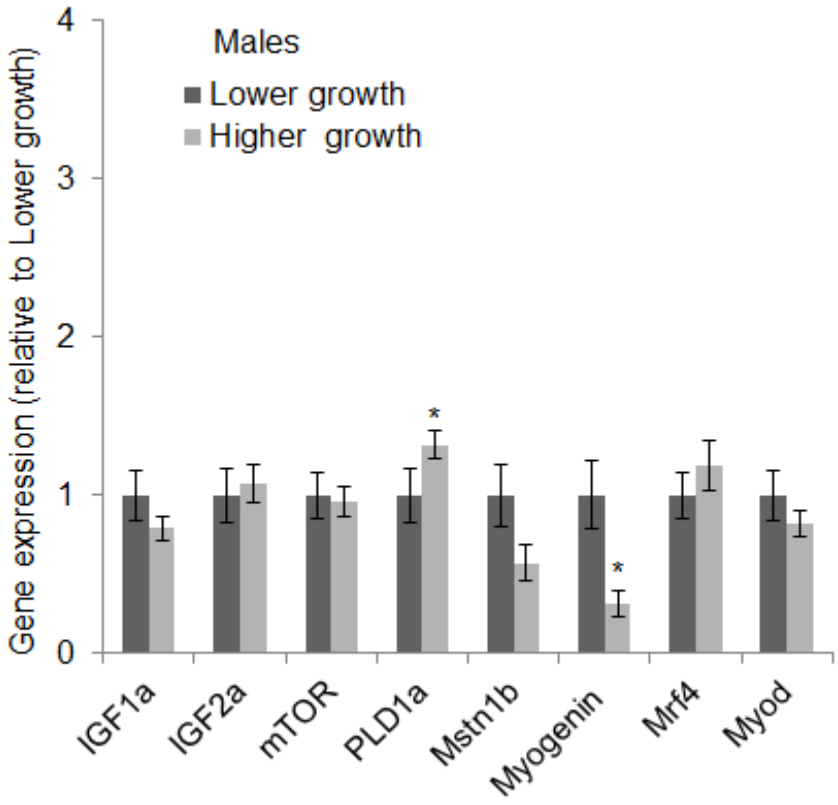

Figure 4.1. Gene expression in muscle of zebrafish males fed with plant protein-based diet. Real time PCR quantification of *Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mstn1b*, *Myogenin*, *Mrf4* and *Myod* in the muscle of higher growth and lower growth zebrafish. Bars indicate the mean and standard errors of 8 data point (8 biological sample x 3 replicate). Significant differences indicated with (*) were determined using Mann-Whitney test ($P<0.05$).

4.3.2. Gene expression in muscle of adult zebrafish of lower and higher growth females fed with plant protein-based diet.

Females with higher growth response to plant protein-based diet had a significant increase in the mRNA expression level ($P<0.05$) for *mTOR* (X1.55 ± 0.27) and *Mstn1b* (X1.82 ± 0.31) in relation to lower growth females (Figure 4.2).

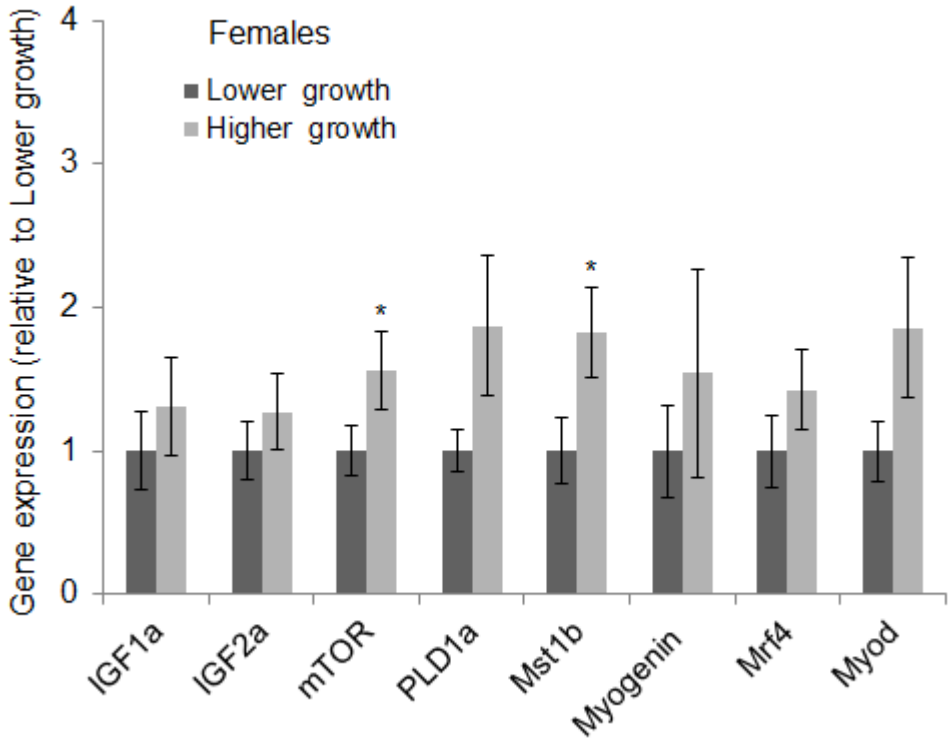

Figure 4.2. Gene expression in muscle of zebrafish females fed with plant protein-based diet. Real time PCR quantification of *Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mstn1b*, *Myogenin*, *Mrf4* and *Myod* in the muscle of higher growth and lower growth zebrafish. Bars indicate the mean and standard errors of 8 data point (8 biological sample x 3 replicate). Significant differences indicated with (*) were determined using Mann-Whitney test ($P<0.05$).

4.4. Discussion

4.4.1. Adult zebrafish of lower and higher growth showed a differential gene expression pattern in *Myogenin* and *Pld1a* in muscle of male zebrafish.

In males with higher growth, *Myogenin* was under-expressed (X0.31 ± 0.08) as compared to males with lower growth, whereas *Pld1a* was over-expressed (X1.31 ± 0.08) (Figure 4.1). In fish, growth muscle occurs through a combination of recruitment of new muscle fibers (hyperplasia) and an increase on the size of existing fibers (hypertrophy) in post-juvenile stages (Johnston, 1999; Mommsen, 2001). However in Zebrafish the number of muscle fibers is established and fixed at the

time of birth and growth is due solely to hypertrophy of fibers already formed (Du, 2004). The decrease of mRNA levels of *Myogenin* in higher growth zebrafish could be explained by the fact that these fish reached their complete growth (hypertrophy) before the time of sampling, when the proliferation and fusion of satellite cell (cells that provide the new nuclei required for skeletal muscle hypertrophy) had slow participation; whereas in lower growth fish these cells could be more active due to possible continuous growth. Similarly, these results can be compared with other studies of rainbow trout (Johansen and Overturf, 2005) and Antarctic fish (*Harpagifer bispini*) (Brodeur et al., 2003) under an different approach (starvation and feeding conditions). The starvation condition decreased the proliferative potential of satellite cells, and subsequent re-feeding linked to growth compensatory effect increased *Myogenin* expression.

In this study mRNA levels of *Pld1a* was over-expressed (X1.31 ± 0.08) in higher growth males as compared to lower growth males (Figure 4.1). The explanation of these results in fish could be reflected by differences in background genetic that underlies the phenotype. In mouse it has been shown that, upon induction of differentiation in C2C12 satellite cells, the expression of *Pld1* was up-regulated and that *Pld1* regulates *mTOR* signaling, and production of *Igf2* (Yoon and Chen, 2008). In this study, non-differential expression in *mTOR* and *Igf2a* between higher growth and lower growth males was observed (Figure 4.2). Our findings suggested that, the genetic background that underlies the fish growth does not influence changes in expression of *mTOR* and *Igf2a* in males. These results suggest that in fish, *Pld1a* could not be an up-stream regulator to *mTOR* as has been demonstrated in mammals. However the phosphorylation and activation of *mTOR* and its related signaling pathways, needs to be examined to major understanding.

4.4.2. Adult zebrafish of lower and higher growth showed a differential gene expression pattern in *Mstn1b* and *mTOR* in muscle of female zebrafish

In females with higher growth, *Mstn1b* (X1.82 ± 0.31) and *mTOR* (X1.55 ± 0.27) were over-expressed as compared to females with lower growth (Figure 4.2). *Mstn* participate as a member of the TGF-β family where the protein function is as a negative regulator of skeletal muscle and growth in mammals. However, *Mstn1 and Mstn2* have been identified in fish, suggesting that several aspects of *mstn* differ significantly from those in mammals. In addition, *Mstn1* had been duplicated in fish producing *Mstn1a* and *Mstn1b* paralogs, where the specific function of these genes has been scarcely documented (Maccatrozzo et al., 2001; Østbye et al., 2001). In this study,

the higher expression of *Mstn1b* in higher growth females probably is related to increment of myoblast-muscle produced in the hypertrophy, as evidences in trout showed that *mstn1b* mRNA was strongly expressed in myoblasts in fish muscle using culture of satellite cells (Seiliez et al., 2012).

The mRNA level of *mTOR* was over-expressed in higher growth females as compared to lower growth (Figure 4.2). Our results suggest that the expression of *mTOR* could be related to size body in higher growth females as compared with lower growth females. A recently study in Arctic charr (*Salvelinus alpinus* L.) evaluated *mTOR* as candidate pathway to understand body-size evolution in dwarf phenotype. Five dwarf and two generalist populations (with ancestral life history and body-size traits), using a standardized manipulation of food intake in a common environment was evaluated. The results showed that throughout refeeding, mTOR was expressed at constitutively higher levels in the generalist compared with dwarf populations (Macqueen et al., 2011). Due that *mTOR* have an essential roles regulating protein synthesis, then our results may be associated with the body size phenotypes of higher and lower growth females.

This approach is a starting point to observe differences in gene expression in muscle among individuals with different phenotypes, even when they were fed with the same diet. This suggests that differences in genetic background allow that some fish can utilize better a diet with higher levels of plant nutrients than other fish. However, further investigations evaluating the same genes in individuals of lower and higher growth in response to fish meal-based diet are needed for better interpretations of results. These genes differentially expressed between phenotypes, could be a good candidate genes to fund genetic variation (SNP) between phenotypes in response to different diets.

4.5. Conclusion

This study showed a differential gene expression pattern related to growth in fish muscle of males and females. In males mRNA level of *Myogenin* was under expressed in higher growth as compared to lower growth males, whereas *Pld1a* was over-expressed. In females the mRNA levels of *mTOR* and *Mstn1b* were over-expressed in higher growth fish as compared to lower growth fish. This difference in gene expression could give account of different genetic background that underlies the growth phenotype in response to plant protein based- diet.

Acknowledgements

The first author received a doctoral fellowship from the Consorcio Empresarial de Genética y Desarrollo Biotecnológico para la Industria Salmonera (Aquainnovo S.A). This work was supported by a fellowship of Apoyo a la Realización de Tesis Doctoral, from Conicyt AT-24091052 and Aquainnovo S.A. PEU thank to laboratorio de Genética y Biotecnología en Acuicultura de la Universidad de Chile.

4.6. References

Adams DC, Rohlf FJ, Slice D E. 2004. Geometric morphometrics: Ten years of progress following the 'revolution', Italian Journal of Zoology 71:5 - 16.

Alami-Durante H, Médale F, Cluzeaud M, Kaushik SJ. 2010. Skeletal muscle growth dynamics and expression of related genes in white and red muscles of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture. 303:50-58.

Brand M, Granato M, Nüsslein-Volhard C. 2002. Keeping and raising zebrafish, in: Zebrafish (C. Nüsslein-Volhard, and R. Dahm, eds.), Oxford University Press, Oxford, pp. 7-37.

Brodeur J C, Calvo J, Johnston I A. 2003, Proliferation of myogenic progenitor cells following feeding in the sub-antarctic notothenioid fish Harpagifer bispinis, J Exp Biol 206:163-169.

Du SJ. 2004. Molecular regulation of fish muscle development and growth in: Molecular Aspects in Fish and Marine Biology (V. Korzh, and Z. Gong eds.), Singapure, pp. 581-611.

Falconer D, Mackay T. 1996. Introduction to quantitative genetics. Pearson. Malaysia.

FAO 2012. El estado mundial de la pesca y la acuicultura. Organización de las Naciones Unidas para la Alimentación y la Agricultura (R. Italy, ed.).

Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF, Kaushik S, Pérez-Sánchez J. 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. 232:493-510.

Johansen KA, Overturf K. 2005. Sequence, conservation, and quantitative expression of rainbow trout Myf5, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 140:533-541.

Johnston IA. 1999. Muscle development and growth: potential implications for flesh quality in fish, Aquaculture 177:99-115.

Krogdahl A, Penn M, Thorsen J, Refstie S, Bakke AM. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquacult.

Res. 41:333-344.

Krogdahl A, Bakke-Mckellep M, Baeverfjord G. 2003. Effects of graded levels of standard soybean meal intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquaculture Nutrition 9:361-371.

Maccatrozzo L, Bargelloni L, Radaelli G, Mascarello F, Patarnello T. 2001. Characterization of the Myostatin Gene in the Gilthead Seabream (*Sparus aurata*): Sequence, Genomic Structure, and Expression Pattern, Marine Biotechnology 3:224-230.

Macqueen DJ, Kristjánsson BK, Paxton CG, Vieira VL, Johnston IA. 2011. The parallel evolution of dwarfism in Arctic charr is accompanied by adaptive divergence in mTOR-pathway gene expression. Mol Ecol. 15:3167-84.

Mommsen TP. 2001. Paradigms of growth in fish, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129:207-219.

Mundheim H, Aksnes A, Hope B. 2004. Growth, feed efficiency and digestibility in salmon (*Salmo salar* L.) fed different dietary proportions of vegetable protein sources in combination with two fish meal qualities. Aquaculture 237:315-331.

Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Forster I, Gatlin DM, Goldburg RJ, Hua K, Nichols PD. 2009. Feeding aquaculture in an era of finite resources, Proceedings of the National Academy of Sciences 106:15103-15110.

NRC, N. R. C. 1993. Nutrient Requeriments of Fish (N. A. Press, ed.), Washington DC, USA.
Østbye TK, Galloway TF, Nielsen C, Gabestad I, Bardal T, Andersen Ø. 2001. The two myostatin genes of Atlantic salmon (*Salmo salar*) are expressed in a variety of tissues, European Journal of Biochemistry 268:5249-5257.

Seiliez I, Sabin N, Gabillard JC. 2012. Myostatin inhibits proliferation but not differentiation of trout myoblasts. Mol Cell Endocrinol 351: 220–226.

Yoon MS, Chen J. 2008. PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. J. Cell Sci. 121:282-289.

Chapter 5. Identification of SNP in differentially expressed genes between lower and higher growth zebrafish fed with plant protein-based diet using RNA-sequencing and growth association study

Manuscript in preparation

Identification of SNP in differentially expressed genes between lower and higher growth zebrafish fed with plant protein-based diet using RNA-sequencing and growth association study

Pilar E. Ulloa[1,2], Gonzalo Rincon[5], Alma Islas-Trejo[5], Cristian Araneda[2], Patricia Iturra[3], Roberto Neira[2,4] and Juan F. Medrano[5]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2]Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile

[3]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70061-7, Santiago, Chile.

[4]AQUAINNOVO S.A., Polpaico 037, Puerto Montt, Chile

[5]Department of Animal Science, University of California-Davis, One Shields Ave, CA 95616, USA

Abstract

The aim objective of this study was to identify SNP in differentially expressed genes in muscle of adult zebrafish with lower and higher growth fed with a plant protein-based diet and performing a growth association study. A population of zebrafish composed from 24 families was fed with a balanced plant protein diet (57.8 % digestible protein, 7.1 % digestible lipids, and 391cal/kg-1 DM energy) from 35 to 98 days post fertilization. From a total of 440 males, five percent in both ends of the normal distribution for weight gain were selected. Total RNA was extracted from individual muscle of eight lower growth fish (average weight = 52 mg) and eight higher growth fish (average weight = 228 mg). RNA-seq libraries were prepared using the Ovation RNA-seq System kit (NuGEN) and sequenced using the Illumina GAII Sequencer. One hundred twenty four genes were differentially expressed between phenotypes (P-value < 0.05 and FDR <0.2) which provided 165 SNP that were genotyped in 240 fish samples for association study. Marker-trait association revealed 5 SNP in *Nars*, *UO-44*, *Acta1b*, *Plac8l1*, and *Lmod2b* respectively. The minor allele from SNP of *Nars* and *UO-44* produced a negative effect on fish growth (-110 mg and -134 mg respectively) and the minor allele from SNP of *Acta1b*, *Plac8l1* and *Tmod2b* produce a positive effect on fish growth (13, 40, 61 mg respectively). These SNP could be used as a step

toward future evaluation applied to species of commercial interest to identify the most efficient individuals for growth in response to a plant protein diet by comparative genomics.

5.1. Introduction

Expansion of aquaculture is seriously limited by reductions in fish meal supply for aquafeeds. Terrestrial alternatives such as plant protein sources have been investigated (FAO, 2012). Recently, some studies have evaluated the transcriptome analysis considering the interaction genotype - diet in Atlantic salmon subject to vegetable oil diet (Leaver et al., 2011, Morais et al., 2011). However, the identification of genetic variants that underlying the growth in response to plant protein diet are not yet identified. From this point of view, nutrigenetics discipline can be useful to investigate the specific genetic variant between individuals that respond in different phenotype to the same diet (Mutch et al., 2005). These genetic variants often occur as single nucleotide polymorphisms (SNP) in coding genes, which could give account of adaptive response of fish to diets with higher levels of plant sources, and influence in the growth expression (Liu and Cordes, 2004, Vignal et al., 2002). They have a codominant inheritance pattern, are adaptable to automated genotyping, and reveal hidden polymorphisms not detected with other markers and methods (Liu and Cordes, 2004, Vignal et al., 2002).

Advances in genetic technologies have opened windows to understand the transcriptome analysis and genetic variation underlying quantitative traits (Cánovas et al., 2010,Wickramasinghe et al., 2011, Wickramasinghe et al., 2012). RNA-sequencing (RNA-seq) is the first sequencing method that allows a quantitative analysis of the entire transcriptome at single base pair resolution (Morozova and Marra, 2008, Mortazavi et al., 2008, Wang et al., 2009). Besides, this technique can detect and quantify various sequence variation (SNP) in coding regions of all expressed genes (Morozova and Marra, 2008, Mortazavi et al., 2008, Wang et al., 2009). This technic could change the practice of candidate gene approach or microarrays experiments, moving toward a more functional understanding of the regulatory networks and pathways that underlying the quantitative traits by finding genetic variation in these genes (Cánovas et al., 2010, Wickramasinghe et al., 2011, Wickramasinghe et al., 2012).

The aim of this study was to identify SNP in differentially expressed genes in muscle of adult zebrafish with lower and higher growth fed with a plant protein-based diet and performing a growth association study in a zebrafish population. We hypothesized that individuals with similar phenotype within a fish population could share the same genetic backgrounds that give account of phenotype in response to plant

protein diet. The identification of differentially expressed genes could represent potential candidate gene to found genetic variation associated to this phenotype (Bamshad et al., 2011).

In this study, zebrafish was used as a model organism for nutrigenetics study. Zebrafish possess advantages compared to farmed fish. Zebrafish are of easier rearing and manipulation in tank of laboratory. They have a short generation intervals and produce a great number of eggs. Zebrafish are omnivores; they can eat a great variety of foods including plant protein diet (Sissener et al., 2010, Kaushik et al., 2011). However, the most powerful genomic resource in zebrafish is the accessibility of its complete genome sequence to performe data analysis by RNA-seq (Grunwald and Eisen, 2002, Orban and Wu, 2008). The major limitation in farmed fish in identifying SNP has been the long generation time (3-4 years in salmonids) to observe segregation of genetic variants associated to growth (Wegrzyn and Ortubay, 2009). Besides, the indispensable genetic information for RNA-seq analysis as the complete genome sequences for farmed fish (Atlantic salmon, Atlantic cod and catfish) are still in progress (Johansen et al., 2009, Davidson et al., 2010 , Lu et al., 2010). Identification of functional polymorphisms in zebrafish and their association with growth in response to a plant protein-based diet is a step toward future evaluation in farmed fish by comparative genomics.

5.2. Material and Methods

5.2.1. Fish rearing

A population of zebrafish composed of 24 experimental families (from single pair mating from no related wild-type strain) was generated in order to identify SNP in a population with a whole genetic variability. Embryoned eggs were incubated at 28° ± 1°C for 7 days until hatching in 9 cm diameter Petri dishes (~100 eggs per Petri dish) (Brand et al., 2002). From 7 to 35 days post fertilization (dpf) each family was reared separately in an aerated 4L container with a density of 25 cm3*fish^{-1}. Larvae were reared under the best physical and chemical parameters of water ($25 \leq T\ °C \leq 28.5$, $7 \leq pH \leq 8$; hardness > 100 mg $CaCO3 \bullet L^{-1}$ and with a photoperiod of 14 h light:10 h dark (Brand et al., 2002). Larvae were fed ad libitum three times daily (at 9:30, 13:30 and 17:30 h) with commercial diet: fine powder feed Sera Micron® (50.2% crude protein, 8.1% crude fat, 4.2% crude fiber and 11.9% ash) and Gold Protein® Micro (49% crude protein, 8% crude fat, 3% crude fiber and 10.5% ash). From 35 to 98 dpf the fish were kept at a density of 350 cm3/fish-1 with optimum physical and chemical parameters of water (Robison et al., 2008). The fish were fed with a balanced plant protein-based diet (mix of soy protein concentrate, corn and wheat gluten meal). The composition in the experimental diet was 57.8 % digestible protein, 7.1

% digestible lipids, and 391cal/kg-1 DM energy. The diet was supplemented with Lysine amino acids to match indispensable amino acid requirements of cyprinids fish (like zebrafish) (NRC, 1993).

5.2.2. Growth trait measurement

For RNA-seq analysis, the fish were selected according to final growth (weight gain, mg) at 98 dpf. In order to select fish in both end of the normal distribution for weight gain (mg), the growth curve was constructed from individual fish data collected at 28 (n=607); 70 (n=342) and 98 (n=779) dpf. Growth curve was evaluated in males and females separately. The fish were fasted for 24 h prior to every sampling event. Each fish was weighed (mg) using a scale with a precision of 0.001 g (Acculab VI-3 mg) and length (mm) was measured from the mouth to the caudal peduncle using digital photography and image analysis software (TPSdig2 v2. 12) (Adams et al., 2004).

5.2.3. Individual selection for RNA-sequencing

In order to avoid potential confound effects in transcriptome analysis between gender, only males (n=440) were considered in this study. Five percent in both ends of weight gain distribution were selected (17 fish with lower growth, average weight = 52 mg and 17 fish with higher growth, average weight = 228 mg). From these fish, eight random fish in each extreme of the weight distribution were selected for RNA-seq libraries preparation (Figure 5.1).

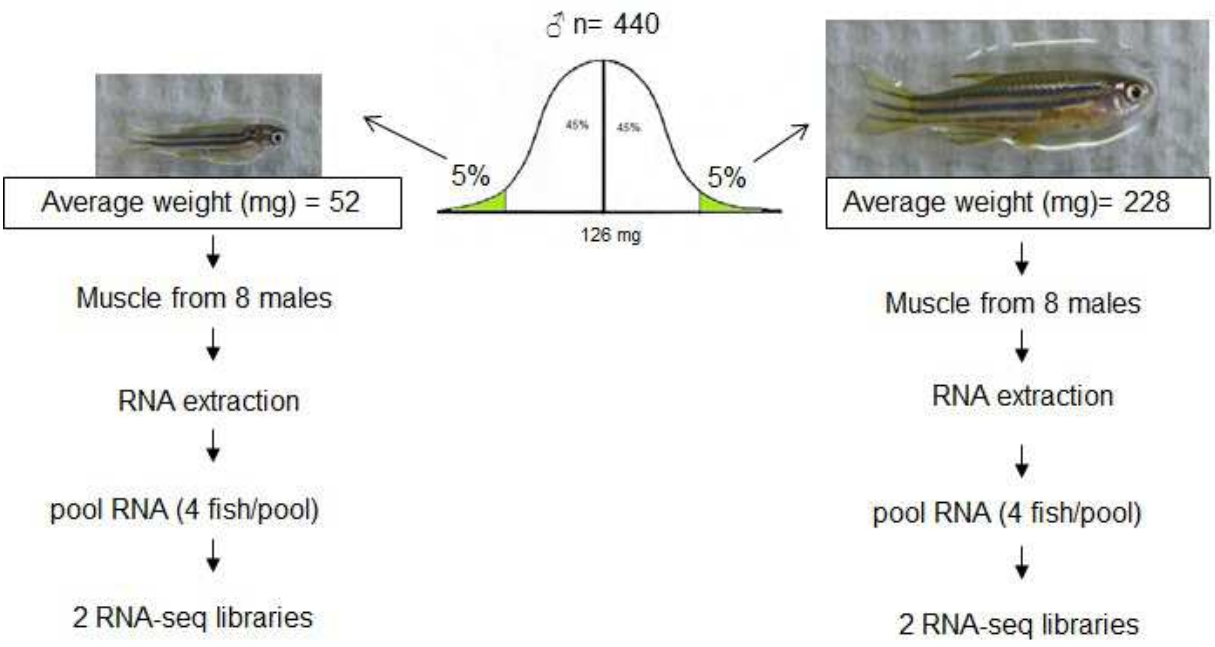

Figure 5.1. Flowchart to fish selection for RNAseq. From a total of 440 males, 17 fish with lower growth (average weight = 52 mg) and 17 fish with higher growth (average weight = 228 mg) were selected in both ends of growth distribution (5% of fish). Muscle sample from 8 random males in each case were selected for RNA extraction. Total RNA was extracted from individual fish and 2 RNA pools (4 fish per pool) per phenotype were performed to prepare RNA-Seq libraries.

5.2.4. RNA extraction

Total RNA was extracted from the muscle of each individual fish. The tissue was homogenized in Trizol® (Invitrogen) following the manufacturer´s protocol. RNA was quantified by ND-1000 spectrophotomer (Fisher Thermo, Wilmington, MA) and the quality and integrity were assessed by the spectrophotometer 260/280 ratio, and by capillary electrophoresis with an Experion bio-analyzer (Bio-Rad, Hercules, CA). All samples were treated with Turbo DNAse I (Ambion, Invitrogen) to remove possible genomic DNA contamination, following the manufacturer´s protocol.

5.2.5. RNA-Sequencing library preparation

Two RNA-seq library preparation were made using Ovation RNA-seq System kit (NuGEN) in RNA pools (4 fish per pool) of lower growth and higher growth fish. Total RNA (100ng) was converted to double-stranded cDNA and posterior SPIA amplification, followed by end repair and adaptor ligation.

The fragments were purified and sequenced at the UC Davis Genome Center DNA Technologies Core Facility using the Illumina Genome Analyzer (GAII).

5.2.6. RNA-seq analysis

Short sequence reads of ~36 - 40 pb were assembled and mapped to the annotated zebrafish reference genome Zv9 (http://www.ensembl.org/index.html) using CLC Genomics workbench 3.7 (CLC Bio, Aarhus, Denmark). Data was normalized by calculating the 'reads per kilobase of exon model per million mapped reads' (RPKM) (Mortazavi et al., 2008). RPKM was ranked as highly expressed (>100 RPKM), medium expressed (100-10 RPKM) and lowly expressed (10-0.2 RPKM). These data were performed on log2-transformed and a correlation between lists of ranked genes was made to verify the reliability of data of libraries. In order to select differentially expressed genes, sequencing reads were merged with annotated genes between lower and higher growth fish. All genes with values greater than ± 2.0-fold change and statistical significant (P-value <0.05 and FRD <0.2) were selected. Fifty four genes were over-expressed in lower growth fish (+ fold-change) and 70 genes were over-expressed in higher growth fish (- fold-change). These genes were candidates to examine functional differences and identify SNP between phenotypes to posterior evaluation in an association study (Figure 5.2).

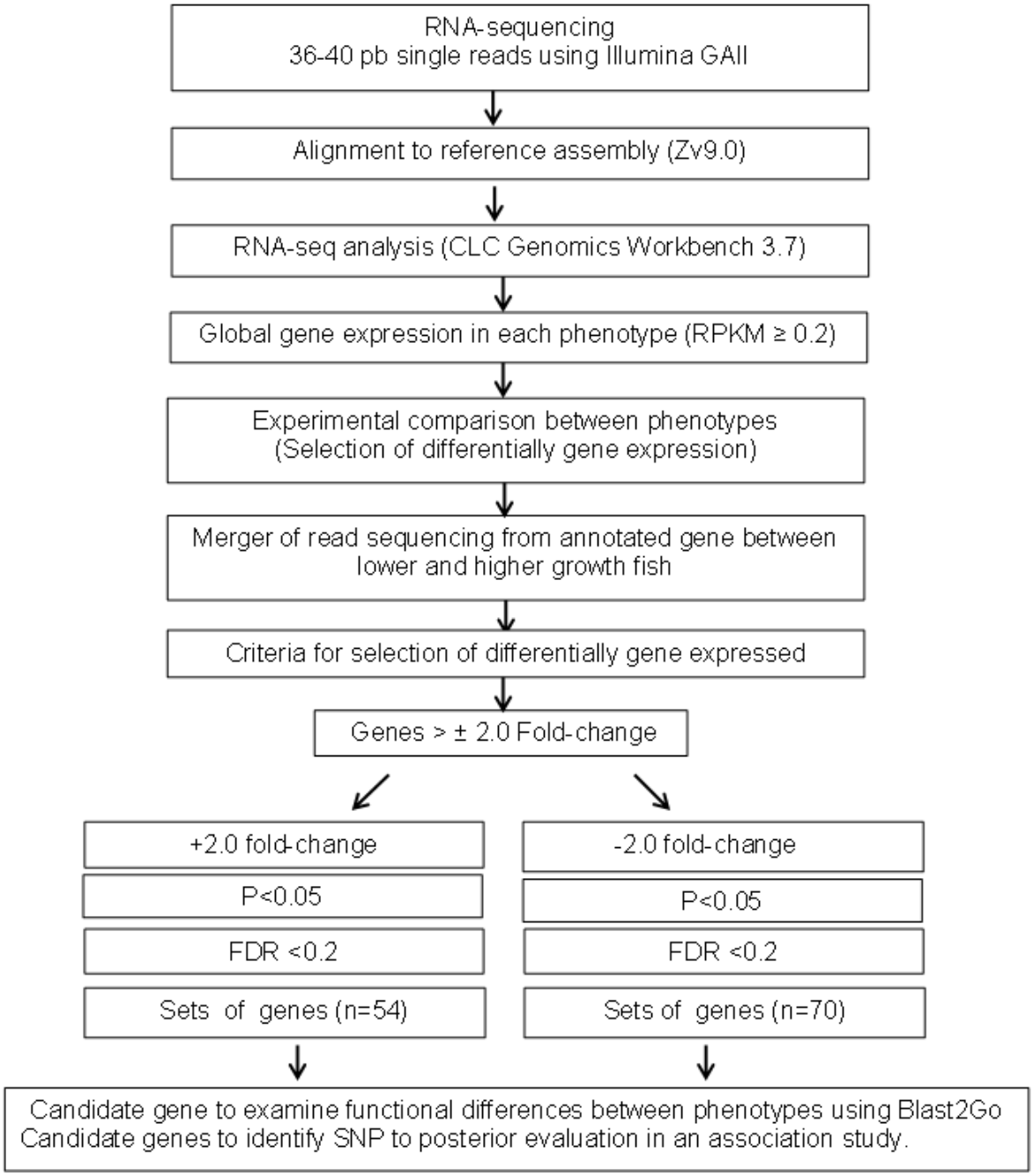

Figure 5.2. Analytical flowchart to study the differential transcriptome between lower and higher growth fish by RNA-seq. An initial global analysis was conducted on genes expressed in muscle of zebrafish followed by an experimental comparison and selection of genes differentially expressed between phenotypes. One set of 54 genes were over-expressed in lower growth fish and another set of 70 genes were over-expressed in higher growth fish. These genes were candidates to examine functional differences by Blast2Go program and identify SNP to posterior evaluation in an association study.

5.2.7. SNP detection by RNA-seq

The total SNP was detected using stringent criteria in order to reduce the rate of detection of false-positive SNP. The following quality and significance filters were applied: (1) the minimum average quality of surrounding bases and minimum quality of the central base were set as 15 and 20 quality score units, respectively; (2) Minimum coverage was set at ten reads; (3) minimum variant frequency or count was set at 20% or two read counts per SNP; and (4) SNP located in the ends (last three bases) were not considered in the analysis due to possible sequencing errors. For genotyping analysis the SNP were selected from the candidate genes (124 genes differentially expressed between phenotype). We assumed that these genes could represent potential candidate to found genetic variation associated to growth due to the functional differences of them between phenotypes. In order to make a new strict SNP selection, a manual curation was performed following criteria: 1) the minimum percentage of allelic variant between phenotype was 60% vs. 40%, 2) SNP location (exon and/or UTR site), and 3) SNP located in the center of reads to avoid possible sequencing errors.

5.2.8. Fish selected for genotyping and association study

In order to evaluate the genetic markers associated to phenotypic growth in the fish population, four individuals from both extreme of the weight curve in each family were selected. A total of 96 fish (24 families x 4 fish) with lower growth (average weight = 79 mg) and 96 fish (24 families x 4 fish) with higher growth (average weight = 225 mg) were selected within the population. In addition, 48 samples of parents representing the 24 families were incorporated in the genotyping analysis. In total, 240 samples were genotyped using allele discrimination by MALDI-TOF mass spectrometry platform (Sequenom MassARRAY(R)) (Neogen/GeneSeek Inc., Lincoln, NE).

Marker-trait association analysis was performed using a linear regression test under an additive model assumption. The linear regression was performed including weight gain (ΔWG) as measured quantitative trait, and gender and families as covariates. Statistical analyses were performed using the genotype association and regression modules from SNP Variation Suite (SVS) Version 7 (Golden Helix Inc., Bozeman, Montana, USA) (Rincón et al., 2009).

The adjusted phenotype (y) was represented with the following equation:

$y = b1x + b0 + e$

Where y was the adjusted phenotype, b1x+b0 represented the model and the error term, e, expressed the random residual effect. The cutoff for significant association was set at p-value ($p<0.05$) and false discovery rate (FDR<0.2).

5.3. Results and Discussion

5.3.1. Overall RNA-seq analysis

RNA sequencing produced an average of 30-35 million of reads for each group of fish. The total of expressed genes in relation to recorded gene (28,401 annotated genes in zebrafish) was in average 17, 227 (~ 60%) and no expressed genes were 1,438 (~ 40%) in fish muscle. The data of RPKM was ranked in each phenotype as highly expressed (>100 RPKM), medium expressed (100-10 RPKM), lowly expressed (10-0.2 RPKM). RNA-seq overall analysis defined 300 genes with high expression in lower growth fish vs. 299 genes with high expression in higher growth; 2,241 genes with medium expression in lower growth fish vs. 2,142 genes with medium expression in higher growth fish; and 14,927 genes with low expression in lower growth fish vs. 14,538 with low expression in higher growth fish (Table 5.1).

Table 5.1. RNA-Seq. gene expression results between different growth fish.

Category[1]	RPKM	Lower growth	Higher growth
Highly expressed genes	> 100	307	299
Medium expressed genes	100 – 10	2,241	2,142
Lowly expressed genes	10-0.2	14,927	14,538
Total expressed genes		17,475	16,979

[1]Genes were annotated using Zv9 with total number of 28,401 genes

The correlation between two replicate RNA-libraries per each phenotype was examined in all ranked categories genes (highly, medium and lowly gene expression). The average correlation was 0.928, 0.782, and 0.794 respectively indicating the reliability of the RNA-seq data.

5.3.2. Differentially expressed genes between lower and higher growth fish

RNA-Seq analysis defined two lists of differentially expressed genes between phenotype (P-value <0.05 and FRD <0.2). A list corresponded to 54 over-expressed genes in lower growth fish (under-expressed in higher growth fish). The other list corresponded to 70 over-expressed genes in higher growth fish (under–expressed in lower growth fish). Both lists corresponded to totally different ID and there were no

correlation between lists (r=0). Gene symbol, gene description, gene ID, statistical parameters (P-value and FDR), fold change and RPKM levels are presented in the supplementary file in Anexo 7 and 8 respectively.

5.3.3. Functional analysis in two sets of differentially expressed genes

In order to observe the most specific GO terms participating in different biological processes between phenotypes, the Fisher's Exact Test with enriched functions was performed by Blas2Go program (Conesa et al., 2005). From 54 expressed genes in lower growth fish, 42% of them are participating in multicellular organismal development, 16% in cell development, 16% in anatomical structure formation involved in morphogenesis, 49% in biological regulation, 30% in cellular component organization, 21% RNA metabolic process and 28% in translation (Figure 5.3A). From 70 expressed genes in higher growth fish, 20% of them are participating in biological process as a sterol biosynthetic process, 22% in cholesterol metabolic process, 25% in generation of precursor metabolites and energy, and 42% in oxidation reduction (Figure 5.3B).

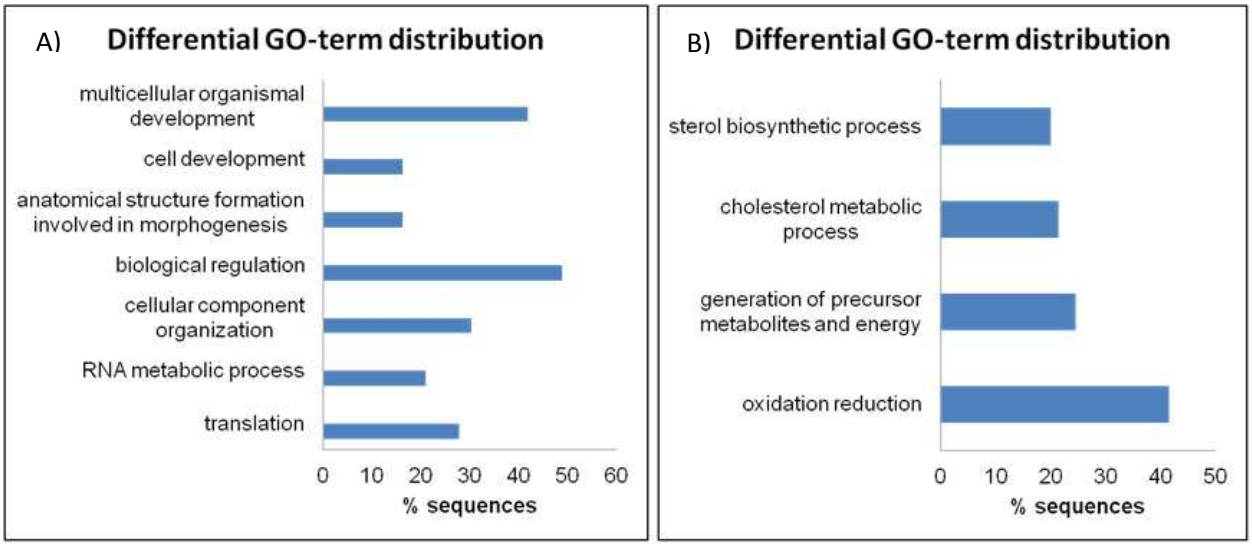

Figure 5.3. Biological process between phenotypes. A) Percentage of 54 genes over-expressed in lower growth fish. B) Percentage of 70 genes over-expressed in higher growth fish.

The Table 5.2 shows the definition of each GO term that are participating in lower growth fish, and Table 5.3 shows the definition of each GO term participating in higher growth fish. The biological processes between phenotypes could reflect differences in fish physiology in relation with metabolism, nutrition and growth development. In lower growth fish, a great percentage of the energy is destined to corporal

growth, and in higher growth fish this energy is destined to sexual maturation and lipid metabolism (Weatherley and Gill, 1987). In the biology of fish growth, the metabolism concept includes chemical reactions that serve to incorporate and build new substances (anabolism) or for destruction (catabolism). In the anabolic reactions, energy is accumulated, while in the catabolic reactions energy is lost. Once food is ingested, it will undergo a series of extracellular digestion processes, participating a series of enzymes in the gut (digestion). They will end up splitting complex substances into simpler ones with release of energy (catabolism). This energy is used by fish for performing their vital functions (movement, respiration, reproduction, etc.) and meets the growth of the organism (Weatherley and Gill, 1987).

Usually variable undergoing change in growth may be measured by physical dimensions (including volume or mass). However, the biological functions related to the content of protein, lipid or other chemical constituent of the body; or caloric (energy) content of the whole body, also are related to growth, as have been showed in the percentage of genes that are involved in this processes (Weatherley and Gill, 1987).

Table 5.2. Descriptions of the most specific Gene Ontology term (GO) participating in over-expressed genes in lower growth fish

GO ID	Term	Description
GO:0007275	Multicellular organismal development	The biological process whose specific outcome is the progression of a multicellular organism over time from an initial condition (e.g. a zygote or a young adult) to a later condition (e.g. a multicellular animal or an aged adult).
GO:0048468	Cell development	The process whose specific outcome is the progression of the cell over time, from its formation to the mature structure. Cell development does not include the steps involved in committing a cell to a specific fate.
GO:0048646	Anatomical structure formation involved in morphogenesis	The developmental process pertaining to the initial formation of an anatomical structure from unspecified parts. This process begins with the specific processes that contribute to the appearance of the discrete structure and ends when the structural rudiment is recognizable. An anatomical structure is any biological entity that occupies space and is distinguished from its surroundings. Anatomical structures can be macroscopic such as a carpel, or microscopic such as an acrosome.
GO:0065007	Biological regulation	Any process that modulates the frequency, rate or extent of any biological process, quality or function.
GO:0016043	Cellular component organization	A process that is carried out at the cellular level which results in the assembly, arrangement of constituent parts, or disassembly of a cellular component.
GO:0016070	RNA metabolic process	The cellular chemical reactions and pathways involving RNA, ribonucleic acid, one of the two main type of nucleic acid, consisting of a long, unbranched macromolecule formed from ribonucleotides joined in 3',5'-phosphodiester linkage.
GO:0006412	Traslation	The cellular metabolic process by which a protein is formed, using the sequence of a mature mRNA molecule to specify the sequence of amino acids in a polypeptide chain. Translation is mediated by the ribosome, and begins with the formation of a ternary complex between aminoacylated initiator methionine tRNA, GTP, and initiation factor 2, which subsequently associates with the small subunit of the ribosome and an mRNA. Translation ends with the release of a polypeptide chain from the ribosome.

Table 5.3. Descriptions of the most specific Gene Ontology term (GO) participating in over-expressed genes in higher growth fish.

GO ID	Term	Description
GO:0006694	steroid biosynthetic process	The chemical reactions and pathways resulting in the formation of steroids.
GO:0008203	cholesterol metabolic process	The chemical reactions and pathways involving cholesterol, cholest-5-en-3 beta-oil, the principal sterol of vertebrates and the precursor of many steroids, including bile acids and steroid hormones. It is a component of the plasma membrane lipid bilayer and of plasma lipoproteins and can be found in all animal tissues.
GO:0006091	generation of precursor metabolites and energy	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived, and any process involved in the liberation of energy from these substances.
GO:0055114	oxidation reduction	The process of removal or addition of one or more electrons with or without the concomitant removal or addition of a proton or protons.

5.3.4. SNP discovery by RNA-seq.

From a total of 225,921 SNP discovered by RNA-seq, a list of 173,186 SNP (according ID and allelic position) were different between phenotypes. However, the candidate genes differentially expressed provided 165 SNP. From them, 83 SNP were selected from 54 over-expressed genes in lower growth fish and 82 SNP were selected from 70 over-expressed in higher growth fish.

5.3.5. SNP association

Marker-trait association results revealed 5 SNP associated with growth (P-value < 0.0001 and FDR <0.2) (Table 5). These SNP are present in *Nars* (novel protein similar to vertebrate asparaginil-tRNA synthetase), *UO-44* or *Cuzd1* (Uncharacterized proteína, represented in zebrafish for CABZ01071923.1), *Acta1b* (actin, alpha skeletal muscle, *Plac8l1* (hypothetical protein, represented in zebrafish for si:dkey-4c2.7) and *Lmod2b* (Leomodulin 2b). The allele substitution effect of SNP belonging to Nars (A/T) and

UO-44 (A/C) produced a negative effects on fish growth (-110 mg and -134 mg respectively) and the allele substitution effect of SNP belonging to *Acta1b* (T/C), *Plac8l1* (T/A) and *Lmod2b* (G/C) produced a positive effect on fish growth (13, 40, 61 mg respectively) (Table 5.4).

Table 5.4. Marker-trait associated with growth phenotype in zebrafish.

Gen	ID gene and SNP position	SNP	Minor allele	Minor allele frequency	p-value	FDR	Allele substitution effect	Amino acid
Nars	ZF61100_17741	A/T	T	0.129	0.0001	0.001	-110	Synonym
UO-44	ZF89599_4308	A/C	C	0.031	0.0056	0.173	-134	Ile500Leu
Acta1b	ZF55618_5089	T/C	T	0.200	0.0033	0.172	13	----------
Plac8l1	ZF87764_4071	T/A	A	0.132	0.0050	0.195	40	----------
Lmod2b	ZF45864_1034	G/C	C	0.223	0.0061	0.158	61	Ser141Thr

Nars

In *Nars*, 11 SNP were identified from RNA-seq (6 in different exons and 5 in UTR site). From them, 3 SNP (2 localized in exon and 1 from UTR site) were genotyped. The rest of SNP were not genotyped due to the proximity between their base pairs. The association study revealed only one SNP (A/T) associated with growth phenotype (reference allele corresponds to (A) and transcript allele corresponds to (T)). This SNP (ZF61100_17741) has a minor allele (T) (minor allele frequency = 0.129) and their substitution effect produced a negative effect on fish growth (-110 mg). RNA-seq analysis revealed that this SNP did not change the amino acid in the protein (Table 5.4). The function of this gene is involved in cellular movement and embryonic development, and participates in aminoacyl-tRNA biosynthesis pathways. Aminoacyl-tRNA is substrates for translation and is pivotal in determining how the genetic code is interpreted as amino acids (Ibba and Söll, 2000).

Zebrafish data base (http://www.ensembl.org/Daniorerio) showed that *Nars* sequence [ID gene: ENSDARG00000061100] has 15 exons and one SNP (A/T) has been previously reported in the exon number 13 (rs4078620, dpSNP http://www.ncbi.nlm.nih.gov/SNP/snp). In order to confirm whether the SNP reported in our study was the same SNP previously described, a sequence alignment (http://www.genome.jp/tools/clustalw/) and nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast) were performed between query sequences. The SNP reported in our study has a 100% similitude with an SNP previously reported (rs4078620). The database showed that the consequence of this variation is a

synonymous coding type (not resulting in an amino acid change; silent mutation) confirming the results obtained by RNA-seq analysis in ours study (Table 5.4). The possible codons to synthetize protein that have been reported were GCC and GCT, codifying to Alanine (Ala) amino acid. The no change of amino acid in the protein suggests there could be others SNP in linkage disequilibrium located in introns or exons closely that could produce this negative effect on phenotypic growth.

UO-44 or *Cuzd1*

In *UO-44*, 15 SNP were identified from RNA-seq (in exons). From them, 4 SNP were not considered in genotyping due to the base pair proximity between them. The association study revealed only one SNP (A/C) associated with growth phenotype (reference allele correspond to (A) and transcript allele correspond to (C)). This SNP (ZF89599_4308) has a minor allele (C) (minor allele frequency = 0.031) and it produced a negative effect on fish growth (-134 mg). RNA-seq analysis revealed that this allelic variant changed the amino acid (Ile500Leu), favoring the protein (Table 5.4). This gene encoded for a transmembrane-associated protein. Primary translation product of the UO-44 gene encodes a peptide of 607 amino acids, which contains a secretory signal sequence, two CUB domains, a zone pellucida domain and transmembrane region (Bork and Beckmann, 1993). CUB domain is a structural motif of approximately 110 residues found almost exclusively in extracellular and plasma membrane-associated proteins, many of which are developmentally regulated (Bork and Beckmann, 1993). These proteins are involved in a diverse range of functions, including complement activation, developmental patterning, tissue repair, cell signaling, inflammation, receptor-mediated endocytosis, and tumor suppression (Abdul Ajees et al., 2006, Perry et al., 2007).

Zebrafish data base (http://www.ensembl.org/Daniorerio) showed that *OU-44* [ID gene: ENSDARG00000089599] has 10 exons and no genetic variations have been previously reported in this gene. This suggests that a new SNP (C/A) has been discovered and the minor allele effect, produce a negative effect on phenotypic growth. However, the sum of effects of other SNP that were no genotyped due to proximity in base pairs and that could be in linkage disequilibrium could be contributing to the negative effect on the growth.

Acta1b

In *Acta1b*, two SNP were identified from RNA-seq (in 3'-UTR site) and both were genotyped. The association study revealed that only one SNP (T/C) was associated with phenotypic growth (reference

allele corresponds to (T) and transcript allele corresponds to (C)). This SNP (ZF55618_5089) has a minor allele (T) (minor allele frequency = 0.200) and it produced a positive effect on fish growth (13 mg). RNA-seq analysis revealed that it did not change the amino acid in the protein (synonym type) (Table 5.4). The gene encodes an skeletal muscle α-actin, which is the predominant actin isoform in adult skeletal muscle, forming the core of the thin filament of the sarcomere (a type of hypertrophy focused to increase protein contractile as actin and myosin) where it interacts with a wide array of proteins, notably myosin of the thick filament, producing force for muscle contraction (Laing et al., 2009).

Zebrafish data base (http://www.ensembl.org/Daniorerio) showed that *Acta1b* sequence [ID gene: ENSDARG00000055618] has 6 exons and 8 SNP have been previously reported in 3'-UTR site (http://www.ensembl.org/Danio_rerio/Variation/). From these SNP one marker (rs179423287, dpSNP, http://www.ncbi.nlm.nih.gov/SNP/snp) has similar variation with the SNP reported in our stuy (C/T). In order to confirm the similitude between both SNP, a sequence alignment (http://www.genome.jp/tools/clustalw/) and nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast) were performed between query sequences. The SNP reported in our study has a 100% similitude with the SNP previously reported (rs179423287). The consequence of this variation was reported as synonymous coding type (not resulting in an amino acid change; silent mutation) confirming the similar result obtained by RNA-seq analysis (Table 5.4). The no change of amino acids in the protein suggests that others SNP present in 3'-UTR or intron site could be in linkage disequilibrium that could contribute with this negative effect.

Plac8l1

Only 1 SNP was identified from RNA-seq in the 3'-UTR site of gene *Plac8l1* and it was genotyped. The association study revealed that this marker was associated with phenotypic growth (reference allele correspond to (T) and transcript allele correspond to (A)). This SNP (ZF87764_4071) has a minor allele (A) (minor allele frequency = 0.132) and produced a positive effect on growth fish (40 mg). RNA-seq analysis revealed that it not produced change in the protein (synonym change) (Table 5.4). The function of this gene is associated with regulators of male germ cell development.

Zebrafish data base (http://www.ensembl.org/Daniorerio) showed that *Plac8l1* sequence (si:dkey-4c2.7) [ID gene: ENSDARG00000087764] has 4 exons, and one SNP has been previously reported in 3'-UTR site (http://www.ensembl.org/Danio_rerio/Variation/). However this SNP (C/G) (rs41002025, dpSNP http://www.ncbi.nlm.nih.gov/SNP/snp) does not have any similitude with SNP reported in our study. This

could suggest that a new variation (T/A) has been discovered in this gene and it produces a positive effect on phenotypic growth.

Lmod2b

In *Lmod2b*, 2 SNP were identified from RNA-seq and only one SNP (G/C) was genotyped due to proximity in base pair between them. The association study revealed that this marker was associated with phenotypic growth (reference allele correspond to (G) and transcript allele correspond to (C)). This marker (ZF45864_1034) has a minor allele (C) (minor allele frequency = 0.223) and it produces a positive effect on fish growth (61 mg). RNA-analysis revealed that it produced a favored change in the protein (Ser141Thr) (Table 5.4). The function of this gene is to participate as a strong filament in muscle cells, actin binding, and tropomyosin binding. Tropomyosin-binding is localized at the pointed end of striated muscle thin filaments and caps the pointed end of tropomyosin-actin filament (Babcock and Fowler, 1994).

Zebrafish data (http://www.ensembl.org/Daniorerio) showed that *Lmod2b* sequence (ID gene: ENSDARG00000045864) has 3 exons and no genetic variants have been previously reported in this gene. This could suggest that a new SNP has been discovered in this gene and the minor allele (C) produces a positive effect on phenotypic growth.

From these allelic variants, two were localized in an exon and correspond to silent mutations and the other three variants were localized in UTR sites which could give account of differential gene expression. These SNP could be molecular markers in disequilibrium linkage with other genes or QTL that underlies the phenotype. This information is relevant because provides SNP that could help us to characterize the most efficient individuals for growth in farmed fish fed with a plant protein diet through comparative genomics. QTL for growth traits (length, body weight and condition factor) have been found in zebrafish, Atlantic salmon, rainbow trout and Arctic charr (*Salvelinus alpinus*) (Baranski et al., 2010, Küttner et al., 2011; Wringe et al., 2010). The identification of conserved genes, sequences or marker (SSR, EST or SNP) between zebrafish and farmed fish could allow knowing the position of any gene that contribute to the phenotype. Synteny among zebrafish and European sea bass (*Dicentrarchus labrax*) (Guyon et al., 2010), Japanese flounder (Castano-Sanchez et al., 2010) turbot (*Scophthalmus maximus*) (Bouza et al., 2012) and Grass carp (*Ctenopharyngodon idella)* (Xia et al., 2010) have been detected. These evidences suggest that the genes reported in this study could be strong candidates for provide a starting point for further characterization of the genetic components underlying growth in fish.

5.4. Conclusion

This study that provides information of five SNP associated to phenotypic growth. Our results revealed that 124 genes were differentially expressed between phenotypes. A number of 54 genes were over-expressed in lower growth fish and 70 genes were over-expressed in higher growth fish. The main biological processes involved in lower growth fish were: protein synthesis, cellular morphology, skeletal and muscle system development, and tissue morphology. The main biological processes involved in higher growth fish were: lipid metabolism, vitamin and mineral metabolism and oxidation reduction. Marker-trait association revealed 5 SNP associated with growth. The allele substitution effect of SNP belonging to *Nars* (A/T) and *UO-44* (A/C) produced a negative effects on fish growth (-110 mg and -134 mg respectively) and the allele substitution effect of SNP belonging to *Acta1b* (T/C), *Plac8l1* (T/A) and *Tmod2b* (G/C) produced a positive effect on fish growth (13, 40, 61 mg respectively). From those SNP, two of them have been previously reported in zebrafish data base (rs4078620 and rs179423287) and three new SNP (UO-44_4308; Plac8l1-4071; Tmod2b-1034) were reported in this study. These SNP are present in chromosome 4, 10, 13, 18 and 21 respectively. These SNP could be used to characterize the most efficient individuals for growth in zebrafish fed with a plant protein-based diet. Besides, provides new candidate genes associated to growth which could be used as molecular markers of regions proximate to other genes or QTL associated to growth.

Acknowledgements

PEU wishes to thank the research group of Animal Genomic Laboratory at the University of California Davis where the RNA-seq experiments were performed, and the UC-Davis Genome Center for his excellent technical expertise to perform Illumina GAII sequencing. PEU received a doctoral fellowship of Consorcio Empresarial de Genética y Desarrollo Biotecnológico para la Industria Salmonera (Aquainnovo S.A). This work was supported by a fellowship of Apoyo a la Realización de Tesis Doctoral from Conicyt AT-24091052, Becas Chile 75110126 and Aquainnovo S.A. PEU also wishes to thank the Programa Convenio Desempeño EVOLUCIONA de la Universidad de la Frontera and its Programa de Doctorado en Ciencias de Recursos Naturales.

5.5. References

Abdul Ajees, A., Gunasekaran, K., Volanakis, J.E., Narayana, S.V.L., Kotwal, G.J. & Krishna Murthy, H.M. (2006). The structure of complement C3b provides insights into complement activation and regulation. *Nature,* 444**:** 221-225.

Adams, D.C., Rohlf, F.J. & Slice, D.E. (2004). Geometric morphometrics: Ten years of progress following the 'revolution'. *Italian Journal of Zoology,* 71**:** 5 - 16.

Babcock, G. & Fowler, V. (1994). Isoform-specific interaction of tropomodulin with skeletal muscle and erythrocyte tropomyosins. *J Biol Chem,* 269**:** 27510-27518.

Bamshad, M.J., Ng, S.B., Bigham, A.W., Tabor, H.K., Emond, M.J., Nickerson, D.A. & Shendure, J. (2011). Exome sequencing as a tool for Mendelian disease gene discovery. *Nat Rev Genet,* 12**:** 745-755.

Baranski, M., Moen, T. & Vage, D. (2010). Mapping of quantitative trait loci for flesh colour and growth traits in Atlantic salmon (Salmo salar). *Genetics Selection Evolution,* 42**:** 17.

Bork, P. & Beckmann, G. (1993). The CUB Domain: A Widespread Module in Developmentally Regulated Proteins. *Journal of Molecular Biology,* 231**:** 539-545.

Bouza, C., Hermida, M., Pardo, B., Vera, M., Fernandez, C., De La Herran, R., Navajas-Perez, R., Alvarez-Dios, J.A., Gomez-Tato, A. & Martinez, P. (2012). An Expressed Sequence Tag (EST)-enriched genetic map of turbot (Scophthalmus maximus): a useful framework for comparative genomics across model and farmed teleosts. *BMC Genetics,* 13**:** 54.

Brand, M., Granato, M. & Nüsslein-Volhard, C. (2002). Keeping and raising zebrafish. IN Nüsslein-Volhard, C. & Dahm, R. (Eds.) *Zebrafish* Oxford: Oxford University Press.

Cánovas, A., Rincon, G., Islas-Trejo, A., Wickramasinghe, S. & Medrano, J. (2010). SNP discovery in the bovine milk transcriptome using RNA-Seq technology. *Mammalian Genome,* 21**:** 592-598.

Castano-Sanchez, C., Fuji, K., Ozaki, A., Hasegawa, O., Sakamoto, T., Morishima, K., Nakayama, I., Fujiwara, A., Masaoka, T., Okamoto, H., Hayashida, K., Tagami, M., Kawai, J., Hayashizaki, Y. & Okamoto, N. (2010). A second generation genetic linkage map of Japanese flounder (Paralichthys olivaceus). *BMC Genomics,* 11**:** 554.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M. & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics,* 21**:** 3674-3676.

Davidson, W., Koop, B., Jones, S., Iturra, P., Vidal, R., Maass, A., Jonassen, I., Lien, S. & Omholt, S. (2010). Sequencing the genome of the Atlantic salmon (*Salmo salar*). *Genome Biology* 11**:** 403.

Fao (2012). El estado mundial de la pesca y la acuicultura. Organización de las Naciones Unidas para la Alimentación y la Agricultura. IN Italy, R. (Ed.).

Grunwald, D.J. & Eisen, J.S. (2002). Headwaters of the zebrafish [mdash] emergence of a new model vertebrate. *Nat Rev Genet,* 3**:** 717-724.

Guyon, R., Senger, F., Rakotomanga, M., Sadequi, N., Volckaert, F.a.M., Hitte, C. & Galibert, F. (2010). A radiation hybrid map of the European sea bass (Dicentrarchus labrax) based on 1581 markers: Synteny analysis with model fish genomes. *Genomics,* 96**:** 228-238.

Ibba, M. & Söll, D. (2000). AMINOACYL-tRNA SYNTHESIS. *Annual Review of Biochemistry,* 69**:** 617-650.

Johansen, S.D., Coucheron, D.H., Andreassen, M., Karlsen, B.O., Furmanek, T., Jørgensen, T.E., Emblem, Å., Breines, R., Nordeide, J.T., Moum, T., Nederbragt, A.J., Stenseth, N.C. & Jakobsen, K.S. (2009). Large-scale sequence analyses of Atlantic cod. *New Biotechnology,* 25**:** 263-271.

Kaushik, S., Georga, I. & Koumoundouros, G. (2011). Growth and body composition of zebrafish (*Danio rerio*) larvae fed a compound feed from first feeding Onward: toward Implications on Nutrient Requirements. *Zebrafish* 8**:** 87-95.

Küttner, E., Moghadam, H., Skúlason, S., Danzmann, R. & Ferguson, M. (2011). Genetic architecture of body weight, condition factor and age of sexual maturation in Icelandic Arctic charr (*Salvelinus alpinus*). *Molecular Genetics and Genomics*, 286**:** 67-79.

Laing, N.G., Dye, D.E., Wallgren-Pettersson, C., Richard, G., Monnier, N., Lillis, S., Winder, T.L., Lochmüller, H., Graziano, C., Mitrani-Rosenbaum, S., Twomey, D., Sparrow, J.C., Beggs, A.H. & Nowak, K.J. (2009). Mutations and polymorphisms of the skeletal muscle α-actin gene (ACTA1). *Human Mutation*, 30**:** 1267-1277.

Leaver, M.J., Taggart, J.B., Villeneuve, L., Bron, J.E., Guy, D.R., Bishop, S.C., Houston, R.D., Matika, O. & Tocher, D.R. (2011). Heritability and mechanisms of n− 3 long chain polyunsaturated fatty acid deposition in the flesh of Atlantic salmon. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 6**:** 62-69.

Liu, Z.J. & Cordes, J.F. (2004). DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, 238**:** 1-37.

Lu, J., Peatman, E., Yang, Q., Wang, S.H., Hu, Z., Reecy, J., Kucuktas, H. & Liu, Z. (2010). The catfish genome database cBARBEL: an informatics platform for genome biology of ictalurid catfish. . *Nucleic Acids Res*, 39**:** D815–D821.

Mcclelland, E.K. & Naish, K.A. (2010). Quantitative trait locus analysis of hatch timing, weight, length and growth rate in coho salmon, Oncorhynchus kisutch. *Heredity*, 105**:** 562-573.

Morais, S., Pratoomyot, J., Taggart, J., Bron, J., Guy, D., Bell, J. & Tocher, D. (2011). Genotype-specific responses in Atlantic salmon (Salmo salar) subject to dietary. *BMC Genomics*, 12**:** 255.

Morozova, O. & Marra, M.A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics*, 92**:** 255-264.

Mortazavi, A., Williams, B.A., Mccue, K., Schaeffer, L. & Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Meth*, 5**:** 621-628.

Mutch, D.M., Wahli, W. & Williamson, G. (2005). Nutrigenomics and nutrigenetics: the emerging faces of nutrition. *The FASEB Journal*, 19**:** 1602-1616.

Nrc, N.R.C. (1993). Nutrient Requeriments of Fish. IN Press, N.A. (Ed.) Washington DC, USA.

Orban, L. & Wu, Q. (2008). Cyprinids. IN Kocher, Thomas, D., Kole & Chittaranjan (Eds.) *Genome Mapping and Genomic in Fishies and Acuatic Animal* Germany Springer.

Perry, S.E., Robinson, P., Melcher, A., Quirke, P., Bühring, H.-J., Cook, G.P. & Blair, G.E. (2007). Expression of the CUB domain containing protein 1 (CDCP1) gene in colorectal tumour cells. *FEBS Letters*, 581**:** 1137-1142.

Reid, D.P., Szanto, A., Glebe, B., Danzmann, R.G. & Ferguson, M.M. (2004). QTL for body weight and condition factor in Atlantic salmon (Salmo salar): comparative analysis with rainbow trout (Oncorhynchus mykiss) and Arctic charr (Salvelinus alpinus). *Heredity*, 94**:** 166-172.

Rincón, G., Islas-Trejo, A., Casellas, J., Ronin, Y., Soller, M., Lipkin, E. & Medrano, J.F. (2009). Fine mapping and association analysis of a quantitative trait locus for milk production traits on Bos taurus autosome 4. *Journal of Dairy Science*, 92**:** 758-764.

Robison, B.D., Drew, R.E., Murdoch, G.K., Powell, M., Rodnick, K.J., Settles, M., Stone, D., Churchill, E., Hill, R.A., Papasani, M.R., Lewis, S.S. & Hardy, R.W. (2008). Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (Danio rerio). *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 3**:** 141-154.

Sanchez-Molano, E., Cerna, A., Toro, M., Bouza, C., Hermida, M., Pardo, B., Cabaleiro, S., Fernandez, J. & Martinez, P. (2011). Detection of growth-related QTL in turbot (Scophthalmus maximus). *BMC Genomics*, 12**:** 473.

Sissener, N.H., Johannessen, L.E., Hevrøy, E.M., Wiik-Nielsen, C.R., Berdal, K.G., Nordgreen, A. & Hemre, G.-I. (2010). Zebrafish (Danio rerio) as a model for investigating the safety of GM feed ingredients (soya and maize); performance, stress response and uptake of dietary DNA sequences. *British Journal of Nutrition*, 103**:** 3-15.

Tave, D. (1993). Genetics for fish hatchery managers. New York

Ulloa, P.E., Iturra, P., Neira, R. & Araneda, C. (2011). Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. *Reviews in Fish Biology and Fisheries***:** 1-18.

Vignal, A., Milan, D., Sancristobal, M. & Eggen, A. (2002). A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution,* 34**:** 275 - 305.

Von Hertell, U., Hörstgen-Schwark, G., Langholz, H.J. & Jung, B. (1990). Family studies on genetic variability in growth and reproductive performance between and within test fish populations of the zebrafish, Brachydanio rerio. *Aquaculture,* 85**:** 307-315.

Wang, Z., Gerstein, M. & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**:** 57-63.

Weatherley, A. & Gill, H. (1987). The biology of fish growth. University of Toronto.

Wegrzyn, D. & Ortubay, S. (2009). Salmonidos en patagonia. Mar del plata, Argentina

Wickramasinghe, S., Hua, S., Rincon, G., Islas-Trejo, A., German, J.B., Lebrilla, C.B. & Medrano, J.F. (2011). Transcriptome Profiling of Bovine Milk Oligosaccharide Metabolism Genes Using RNA-Sequencing. *PLoS ONE,* 6**:** e18895.

Wickramasinghe, S., Rincon, G., Islas-Trejo, A. & Medrano, J. (2012). Transcriptional profiling of bovine milk using RNA sequencing. *BMC Genomics,* 13**:** 45.

Wright, D., Nakamichi, R., Krause, J. & Butlin, R. (2006). QTL Analysis of Behavioral and Morphological Differentiation Between Wild and Laboratory Zebrafish (<i>Danio rerio). *Behavior Genetics,* 36**:** 271-284.

Wringe, B., Devlin, R., Ferguson, M., Moghadam, H., Sakhrani, D. & Danzmann, R. (2010). Growth-related quantitative trait loci in domestic and wild rainbow trout (Oncorhynchus mykiss). *BMC Genetics,* 11**:** 63.

Xia, J., Liu, F., Zhu, Z., Fu, J., Feng, J., Li, J. & Yue, G. (2010). A consensus linkage map of the grass carp (Ctenopharyngodon idella) based on microsatellites and SNPs. *BMC Genomics,* 11**:** 135.

Chapter 6. General discussion

6. General discussion

In this thesis we examine growth response and evaluated gene expression related to growth (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Myostatin1b*) in muscle of adult zebrafish (*Danio rerio*) fed with plant and fishmeal protein-based diets (Chapter III). In this chapter we used a new experimental design for nutrigenomics studies in zebrafish. In brief, the growth phenotype was measured in a fish population of 24 experimental families. Each family was split to create two fish populations with similar genetic background. In order to examine the effect of PP diet on gene expression, individuals of average weight gain in both populations were selected. In order to examine the effect of familiar variation on gene expression, the three families were evaluated separately. This study demonstrated that PP diet reduce fish growth compared with FM diet. These results could be compared with findings in other studies in zebrafish fed with different formulated diets. For example, Robison et al. (2008) demonstrated that manipulation of dietary carbohydrate levels (0, 15, 25 and 35%) had a significant effect on body weight and condition factor. In another study, zebrafish were fed with different sources of plant protein (soy bean + corn genetically modified (GM) and non-GM). Fish fed with GM corn exhibited significantly better growth as compared with fish fed with non-GM corn (Sissener et al., 2010). Our results can also be compared with studies in farmed fish fed with different percentages PP replacement of FM. Our results are consistent with findings in common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and rainbow trout, which showed a marked reduction in growth when 6–19 g alevins were fed with a diet of 75–100% FM replacement (Alami-Durante et al., 2010; Fontaínhas-Fernandes et al., 1999; Médale et al., 1998; Pongmaneerat et al., 1993; Vilhelmsson et al., 2004). In this study, growth traits were evaluated in zebrafish from alevin to adult stages (28 to 98 dpf) in populations with similar genetic backgrounds. Therefore our results may be attributed principally to differences between diets. The decreased growth in zebrafish fed with PP diet could be related to antinutritional factors in the plant protein diet (wheat, corn and soy concentrate) as well as limited availability of essential amino acids, such as arginine (2.9%), lysine (2.5%) and methionine (0.7). On the other hand, discordant results have also been reported in70–83 g juvenile rainbow trout, which showed similar growth in fish fed with high replacement of FM (66-100%) with soy protein concentrate (Kaushik et al., 1995; Olli et al., 1994). These differences between studies may be attributed to the biological developmental stage at the beginning of the experimental period.

Differences at 28 dpf in initial weight among offspring of different families were likely the product of inter-family genetic differences within the population. At 70 and 98 dpf some families tend to maintain their rank from initial weight (28 dpf). However, in most families fed with FM and PP diet, initial weight

gain ranking is not maintained. The families with the greatest increases in weight gain fed when with FM diet did not maintain the same ranking when fed with PP diet. This indicates that the genetic components underlying growth phenotype in each family produced a different physiological response depending on the plant or fishmeal protein-based diet. These could reflect genotype environment interaction.

In relation to gene expression in response to diets, our results showed that *Igf2a*, *Myogenin* and *Mrf4* were modulated by plant nutrients in muscle of zebrafish males. *Igf2a* belonging to somatotropic axis was under-expressed (X0.71 ± 0.09) in fish fed with a PP diet as compared with gene expression of males fed with the FM diet. This Igf2a expression pattern could be explained by lower lysine levels in PP diet as compared with FM diet (2.5 vs. 4.2 respectively), as has been shown in Atlantic salmon when fish fed with low lysine (2.9 g/16 g N) showed a 2.6-fold decrease in *Igf2a* mRNA levels compared to those fed with medium lysine levels (4.9 g/16 g N) (Hevroy et al., 2007). *Myogenin* (X5.0 ± 0.67) and *Mrf4* (X3.42 ± 0.56) were over-expressed in males fed with a PP diet as compared with FM diet. *Myogenin* and *Mrf4* are essential factors involved in the late steps of myogenesis and in hypertrophy (increase in size of existing muscle fibers) and hyperplasia (recruitment of new muscle fibers) (Johnston et al., 2011). They are involved in the differentiation process, generating myotubes which can fuse to form short myoblast-myotubes, and fusion events, producing fiber maturation and hypertrophy (Johnston, 2006). The nutritional regulation of muscle is poorly documented in fish. A single report in rainbow trout showed that the substitution of FM with graded levels of PP (50%, 75%, 100% replacement) in diets did not modify the expression of *Myogenin* in fish muscle (Alami-Durante et al., 2010). This difference in gene expression in response to PP diet between species could be due to different processes involved in muscle growth (hypertrophy in zebrafish and hyperplasia and hypertrophy in rainbow trout) (Du et al., 2004; Johnston et al., 2008). Similarly, these results can be compared with other studies of rainbow trout (Johansen and Overturf, 2005) and Antarctic fish (*Harpagifer bispini*) (Brodeur et al., 2003) under a different approach (starvation and feeding conditions). The starvation condition decreased the proliferative potential of satellite cells participating in hypertrophy and the re-feeding condition increased the activity of satellite cells produced by compensatory growth. The *Myogenin* expression was increased in re-feeding condition as compared with starvation condition. These results suggest that *Myogenin* is a candidate gene associated with growth and it is greatly modulated by nutrition.

In females, there were no statistical differences in gene expression by diet for any of the eight genes studied (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mstn1b*, *Myogenin*, *Mrf4*, *Myod*). The presence of sexual dimorphism in gene expression has been also described in zebrafish liver and brain (Robison et al., 2008; Santos et al., 2008). However, our results show that even when all eight genes were expressed in the

muscle of males and females, differential expression of *Myogenin*, *Mrf4* and *Igf2a* in response to PP diet was remarkable only in males. That would suggest that males were more affected by PP diets than females.

In order to evaluate the familiar effect on gene expression, the data were analyzed for each family separately. The familiar variation effect was observed in males and females among families. In males, *Myogenin* and *Mrf4* were over-expressed in response to PP diet in all three families selected. This suggests that the expression of these genes in males was modulated principally by the plant ingredients rather than genetic differences among families. *Igf2a*, *Igf1a*, *Pld1a* and *Mstn1b* were differentially expressed in the families 4 and 23. In females, *Igf1a*, *Myogenin*, *Myod*, *mTOR* and *Pld1a* were expressed differentially by diet in the same families (4 and 23). These results suggest that differences in gene expression may be influenced not only by dietary components but also by genetic differences that underlie each family. Therefore it is important to pay attention to both sources of variation in explaining the modulation of gene expression in nutrigenomics studies. These results could confirm that the individuals from the families 4 and 23 were more affected by PP diet even when their growth was not modified.

On the other hand, considering that the growth is a trait that varies among individuals in a population, we examined the expression of genes related to growth (*Igf1a*, *Igf2a*, *mTOR*, *Pld1a*, *Mrf4*, *Myod*, *Myogenin* and *Mst1b*) in muscle of adult lower and higher growth zebrafish fed with plant protein-based diet (Chapter IV). In males with higher growth, *Myogenin* was under-expressed (X0.31 ± 0.08) in relation to males with lower growth, whereas *Pld1a* was over-expressed (X1.31 ± 0.08). *Myogenin* is an essential factor involved in the late steps of myogenesis and also is involved in the process of hypertrophy and hyperplasia (Atchley et al., 1994; Hinits et al., 2007; Johansen and Overturf, 2005). The decrease of mRNA levels of *myogenin* in high growth zebrafish could be explained by the fact that these fish reached their complete growth (hypertrophy) before the time of sampling, when the proliferation and fusion of satellite cell (cells that provide the new nuclei required for skeletal muscle hypertrophy) had slow participation; whereas in low growth fish these cells could be more active due to possible continuous growth. The explanation of these results in fish could be reflected by differences in background genetic that underlies the phenotype. In mouse it has been shown that upon induction of differentiation in C2C12 satellite cells, the expression of *Pld1* was up-regulated and that *Pld1* regulates *mTOR* signaling, and production of *Igf2* (Yoon and Chen, 2008). In this study, non-differential expression in *mTOR* and *Igf2a* between higher growth and lower growth males was observed. Our findings suggested that, the genetic background that underlies the fish growth does not influence changes in expression of *mTOR* and *Igf2a* in

males. These results suggest that in fish, *Pld1a* could not be an up-stream regulator to *mTOR* as has been demonstrated in mammals. However the phosphorylation and activation of *mTOR* and its related signaling pathways, needs to be examined to major understanding.

In females with higher growth, *Mstn1b* (X1.82 ± 0.31) and *mTOR* (X1.55 ± 0.27) were over-expressed in relation to females with lower growth. MSTN is a member of the TGF-β family that functions as a negative regulator of skeletal muscle development and growth in mammals. However, in fish *Mstn1* and *Mstn2* have been identified, suggesting that several aspects of *Mstn* differ significantly from those in mammals. In addition, *Mstn1* had been duplicated producing *Mstn1a* and *Mstn1b* paralogs, where the specific function of these genes has been scarcely documented in fish (Maccatrozzo et al., 2001; Ostbye et al., 2001).. In this study, the higher expression of *Mstn1b* in higher growth females probably is related to increment of myoblast-muscle produced in the hypertrophy. Recently evidences in trout using culture of satellite cells showed that *Mstn1b* mRNA was strongly expressed in myoblasts in fish muscle (Seiliez et al., 2012).

The levels of mRNA for *mTOR* were over-expressed in high growth females in relation to lower growth. This difference could be due to genetic component that underlies the body size, although the molecular mechanisms in this pathway have been poorly understood in fish. There are evidence that *mTOR* is differentially expressed in fish under fasting and re-feeding in trout and zebrafish (Craig and Moon, 2011, Skiba-Cassy et al., 2009). However, a recently study in Arctic charr (*Salvelinus alpinus* L.) evaluated *mTOR* as candidate pathway to understand body-size evolution in dwarf phenotype. Five dwarf and two generalist populations (with ancestral life history and body-size traits), using a standardized manipulation of food intake in a common environment was evaluated. The results showed that throughout refeeding, mTOR was expressed at constitutively higher levels in the generalist compared with dwarf populations (Macqueen et al., 2011). Due that *mTOR* have an essential roles regulating protein synthesis, then our results may be associated with the body size phenotypes of higher and lower growth females.

In order to select potential candidate genes to find genetic variation (SNP) that could be associated to growth phenotype, we identified all genes with different expression in muscle of lower and higher growth fish by RNA-seq technology (Chapter V). This analysis was performed only in males in order to avoid confounding effect of sexual dimorphism on transcriptional levels. Analysis revealed 124 genes differentially expressed between phenotypes which provided 165 SNP that were genotyped in 240 samples. Analysis revealed five SNP associated with growth, each one of them was located in *Nars*, *OU-44*, *Acta1b*, *Plac8l1*, *Lmod2a* respectively. *Nars* is involved in cellular movement and embryonic

development, and participates in aminoacyl-tRNA biosynthesis pathways (Ibba and Söll, 2000). *OU-44* is involved in developmental patterning, tissue repair, cell signaling, inflammation, receptor-mediated endocytosis, and tumor suppression (Abdul Ajees et al., 2006, Perry et al., 2007). *Acta1b* encodes an skeletal muscle α-actin, which is the predominant actin isoform in adult skeletal muscle, forming the core of the thin filament of the sarcomere (a type of hypertrophy focused to increase protein contractile as actin and myosin) where it interacts with a wide array of proteins, notably myosin of the thick filament, producing force for muscle contraction (Laing et al., 2009). *Plac8l1* is associated with regulators of male germ cell development and *Lmod2b* participates as a strong filament in muscle cells, actin binding, and tropomyosin binding localized at the pointed end of striated muscle thin filaments (Babcock and Fowler, 1994). The minor allele from SNP of *Nars* and *UO-44* produced a negative effect on fish growth (-110 mg and -134 mg respectively) and the minor allele from SNP of *Acta1b*, *Plac8l1* and *Tmod2b* produce a positive effect on fish growth (13, 40, 61 mg respectively). From these allelic variants, two were localized in an exon and correspond to silent mutations and the other three variants were localized in UTR sites which could give account of differential gene expression. These SNP could be molecular markers in disequilibrium linkage with other genes or QTL that underlies to the phenotype. This information is relevant firstly because provides SNP that could help us to characterize the most efficient individuals for growth in farmed fish fed with a plant protein diet through comparative genomics. QTL for growth traits (length, body weight and condition factor) have been found in zebrafish, Atlantic salmon, rainbow trout and Arctic charr (*Salvelinus alpinus*) (Baranski et al., 2010, Küttner et al., 2011; Wright et al., 2006; Wringe et al., 2010). The identification of conserved genes, sequences or marker (SSR, EST or SNP) between zebrafish and farmed fish could allow knowing the position of any gene that contribute to the phenotype. Synteny among zebrafish and European sea bass (*Dicentrarchus labrax*) (Guyon et al., 2010), Japanese flounder (Castano-Sanchez et al., 2010) turbot (*Scophthalmus maximus*) (Bouza et al., 2012) and Grass carp (*Ctenopharyngodon idella)* (Xia et al., 2010) have been detected. These evidences suggest that the genes reported in this study could be strong candidates for provide a starting point for further characterization of the genetic components underlying growth in fish.

References

Alami-Durante H, Médale F, Cluzeaud M, Kaushik SJ. 2010. Skeletal muscle growth dynamics and expression of related genes in white and red muscles of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture 303:50-8.

Abdul Ajees A, Gunasekaran K, Volanakis JE, Narayana SVL, Kotwal GJ, Krishna Murthy HM. 2006. The structure of complement C3b provides insights into complement activation and regulation. Nature. 444: 221-225.

Atchley WR, Fitch WM, Bronner-Fraser M. 1994. Molecular evolution of the MyoD family of transcription factors. Proc. Natl. Acad. Sci. USA, 91, 11522-11526.

Babcock G. & Fowler V. 1994. Isoform-specific interaction of tropomodulin with skeletal muscle and erythrocyte tropomyosins. J Biol Chem, 269: 27510-27518.

Baranski M, Moen T, Vage D. 2010. Mapping of quantitative trait loci for flesh colour and growth traits in Atlantic salmon (*Salmo salar*). Genetics Selection Evolution 42: 17.

Bouza C, Hermida M, Pardo B, Vera M, Fernandez C, De La Herran R, Navajas-Perez R, Alvarez-Dios JA, Gomez-Tato A, Martinez P. 2012. An Expressed Sequence Tag (EST)-enriched genetic map of turbot (*Scophthalmus maximus*): a useful framework for comparative genomics across model and farmed teleosts. BMC Genetics. 13: 54.

Brodeur, J.C., Calvo, J., Johnston, I.A., 2003. Proliferation of myogenic progenitor cells following feeding in the sub-antarctic notothenioid fish Harpagifer bispinis. J Exp Bio. 206, 163-169.

Castano-Sanchez C, Fuji K, Ozaki A, Hasegawa O, Sakamoto T, Morishima K, Nakayama I, Fujiwara A, Masaoka T, Okamoto H, Hayashida K, Tagami M, Kawai J, Hayashizaki Y, Okamoto N. 2010. A second generation genetic linkage map of Japanese flounder (Paralichthys olivaceus). BMC Genomics. 11: 554.

Craig PM, Moon TW. 2011. Fasted zebrafish mimic genetic and physiological responses in mammals: a model for obesity and diabetes? Zebrafish 3: 109-117.

Du SJ: Molecular regulation of fish muscle development and growth. In: Korzh V, Gong Z, (eds). Molecular Aspects in Fish and Marine Biology, Singapure, 2004.

Fontaínhas-Fernandes A, Gomes E, Reis-Henriques MA, Coimbra J. 1999. Replacement of Fish Meal by Plant Proteins in the Diet of Nile Tilapia: Digestibility and Growth Performance. Aquaculture International 7:57-67.

Guyon R, Senger F, Rakotomanga M, Sadequi N, Volckaert FAM, Hitte C, Galibert F. 2010. A radiation hybrid map of the European sea bass (*Dicentrarchus labrax*) based on 1581 markers: Synteny analysis with model fish genomes. Genomics. 96: 228-238.

Hevrøy EM, El-Mowafi A, Taylor RG, Olsvik PA, Norberg B, Espe M. 2007. Lysine intake affects gene expression of anabolic hormones in atlantic salmon, *Salmo salar*. General and Comparative Endocrinology. 152:39-46.

Hinits Y, Osborn DS, Carvajal JJ, Rigby PW, Hughes SM, 2007. Mrf4 (myf6) is dynamically expressed in differentiated zebrafish skeletal muscle. Gene Expression Patterns. 7: 738-745

Ibba M and Söll, D. 2000. AMINOACYL-tRNA SYNTHESIS. Annual Review of Biochemistry. 69: 617-650.

Johansen KA, Overturf K, 2005. Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 144:119-127.

Johnston IA. 2006. Environment and plasticity of myogenesis in teleost fish. The Journal of Experimental Biology 209: 2249-2264

Johnston I, Macqueen D, Watabe S. 2008. Molecular biotechnology of development and growth in fish muscle. In: Fisheries for global welfare and environment. Tsukamoto K, Kawamura T, Takeuchi T, Douglas Beard T, Kaiser M (eds), 5th World Fisheries Congress, Scotland.

Johnston IA, Bower NI, Macqueen DJ. 2011. Growth and the regulation of myotomal muscle mass in teleost fish Journal of Experimental Biology 214: 1617-28.

Küttner E, Moghadam H, Skúlason S, Danzmann R, Ferguson M. 2011. Genetic architecture of body weight, condition factor and age of sexual maturation in Icelandic Arctic charr (*Salvelinus alpinus*). Molecular Genetics and Genomics. 286: 67-79.

Laing NG, Dye DE, Wallgren-Pettersson C, Richard G, Monnier N, Lillis S, Winder TL, Lochmüller H, Graziano C, Mitrani-Rosenbaum S, Twomey D, Sparrow JC, Beggs AH, Nowak KJ. 2009. Mutations and polymorphisms of the skeletal muscle α-actin gene (*ACTA1*). Human Mutation 30: 1267-1277.

Macqueen DJ, Kristjánsson BK, Paxton CG, Vieira VL, Johnston IA. 2011. The parallel evolution of dwarfism in Arctic charr is accompanied by adaptive divergence in mTOR-pathway gene expression. Mol Ecol. 15:3167-84.

Maccatrozzo L, Bargelloni L, Radaelli G, Mascarello F, Patarnello T, 2001. Characterization of the Myostatin Gene in the Gilthead Seabream (*Sparus aurata*): Sequence, Genomic Structure, and Expression Pattern. Mar. Biotechnol. 3: 224-230.

Médale F, Boujard T, Vallée F, Blanc D, Mambrini M, Roem A, et al. 1998: Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (*Oncorhynchus mykiss*) fed increasing dietary levels of soy protein concentrate. Aquatic Living Resources 11:239-46.

Ostbye TK, Galloway TF, Nielsen C, Gabestad I, Bardal T, Andersen O. 2001. The two myostatin genes of Atlantic salmon (Salmo salar) are expressed in a variety of tissues. Eur. J. Biochem. 268: 5249-5257.

Perry SE, Robinson P, Melcher A, Quirke P, Bühring HJ, Cook GP, Blair GE. 2007. Expression of the CUB domain containing protein 1 (CDCP1) gene in colorectal tumour cells. FEBS Letters. 581: 1137-1142.

Pongmaneerat J, Watanabe T, Takeuchi T, Satoh T. 1993. Use of different protein meals as partial or total substitution for fish meal in carp diets. Tokyo, JAPON: Nippon Suisan Gakkai.

Robison BD, Drew RE, Murdoch GK, Powell M, Rodnick KJ, Settles M. 2008. Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 3:141-54.
Santos E, Kille P, Workman V, Paull G, Tyler Ch. 2008. Sexually dimorphic gene expression in the brains of mature zebrafish. Comp Biochem Physiol A Mol Integr Physiol. 149:314-24.

Seiliez I, Sabin N, Gabillard JC. 2012. Myostatin inhibits proliferation but not differentiation of trout myoblasts. Mol Cell Endocrinol 351: 220–226.

Sissener NH, Johannessen LE, Hevrøy EM, Wiik-Nielsen CR, Berdal KG, Nordgreen A, Hemre GI, 2010. Zebrafish (*Danio rerio*) as a model for investigating the safely of GM feed ingredients (soya and maize) performance, stress response and uptake of dietary DNA sequences. Br. J. Nutr.103: 3-15.

Skiba-Cassy S, Lansard M, Panserat S, Médale F. 2009. Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297: R1421-R1429.

Wringe B, Devlin R, Ferguson M, Moghadam H, Sakhrani D, Danzmann R. 2010. Growth-related quantitative trait loci in domestic and wild rainbow trout (*Oncorhynchus mykiss*). BMC Genetics. 11**:** 63.

Wright D, Nakamichi R, Krause J, Butlin R. 2006. QTL Analysis of Behavioral and Morphological Differentiation Between Wild and Laboratory Zebrafish (*Danio rerio*). Behavior Genetics. 36: 271-284.

Vilhelmsson OT, Martin SAM, Médale F, Kaushik SJ, Houlihan DF. 2004. Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (*Oncorhynchus mykiss*). British Journal of Nutrition 92:71-80

Xia J, Liu F, Zhu Z, Fu J, Feng J, Li J, Yue G. 2010. A consensus linkage map of the grass carp (*Ctenopharyngodon idella*) based on microsatellites and SNPs. BMC Genomics 11: 135.

Yoon MS, Chen J. 2008. *PLD* regulates myoblast differentiation through the mTOR-IGF2 pathway. J. Cell Sci. 121, 282-289.

Chapter 7. General Conclusions

7. General Conclusions

Taking into account the main results in this thesis, it can be concluded that:

- The expression of *Myogenin*, *Mrf4* and *Igf2a* was modulated by plant protein diet in males. While in females, the expression of eight candidate genes associated with growth were not modulate by plant protein based-diet. This shows clearly that the gene expression regulation in response to diet is independent in males and females.

- The effect of familiar variation on gene expression was observed among families. That suggests that there were families more affected to high levels of plant protein even when the individuals did not modify their growth.

- The expression of *Myogenin* was under-expressed in the muscle of males with higher growth as compared to lower growth and *Pld1a* was over-expressed. While, the expression of *mTOR* and *Mstn1b* were over-expressed in females. These differences in gene expression between phenotypes could give account of different development stages and genetic background that underlies the growth.

- This study provides five SNP associated with growth, each one of them was located in *Nars*, *OU-44, Acta1b, Plac8l1, and Lmod2a* respectively. These SNP could be used to characterize the most efficient individuals for growth in zebrafish fed with a plant protein-based diet. Besides, provides new candidate genes associated to growth to evaluate in future research.

Outlook

The information generated in this thesis has enhanced our understanding about the effect of plant protein sources on gene expression related to growth and have provided SNP associated to growth which could be used to characterize the most efficient individuals fed with a plant protein-based diet. However it is necessary to integrate in systems biology not only the transcriptomics, but also the proteomic, and metabolomic information to give a more complete knowledge about complex trait as growth. The studies of transcriptomics give light about the gene expression affected by stimule in a given time. Proteomics show the function of the protein in the organism and metabolomic shows the metabolites affecting the

physiological response in the organism. This last tool can be used to perform a screening and to identify biochemical markers in fish fed with plant protein diets.

In relation to future works, would be interesting measured the effect of plant protein on transcriptome in brain tissue, and select genes related to perception of the senses in fish (nose, eye, mouth) due that the perception of food is governed by hypothalamic axis. These genes could be new candidate genes to identify SNP. On the other hand, RNA-seq. could be used to evaluate transcriptome in various tissues (brain, intestine, liver) in fish fed with plant and fishmeal protein-based diet. In such a way to identify metabolic pathway committed with plant nutrients and to design a panel of genes that are regulated differentially by diets and between tissues. That could be useful tools for future studies in nutritional genomics in farmed fish through genomics comparative.

ANEXOS

Anexo 1.

Water quality parameters during rearing of fish. Values are mean ± standard deviation.

Parameter	Larval rearing (7 to 35 dpf)	Juvenile rearing (35 to 98 dpf)	Optimum ranges	Reference
pH	7.67 ± 0.13	7.73±0.13	7.0 – 8.0	Brand et al., 2002
Oxigen (mg $O_2 \cdot L^{-1}$)	7.30 ± 0.62	6.95 ±0.65	~ 7.8 at 28°C	Lawrence, 2007
Temperature (°C)	25.71 ± 1.25	28.10±0.45	24 – 28°C	Brand et al., 2002
Hardness ($CaCO_3$; mg$\cdot L^{-1}$)	141.3 ± 23.90	224.94 ±12.05	75-200	Brand et al., 2002
Nitrate (NO_3^- mg $\cdot L^{-1}$)*	69.6 ± 82.7	57.32±34.28	> 200	Lawrence, 2007
Nitrite (NO_2^- mg $\cdot L^{-1}$)*	5 ± 4.92	0.6±0.69	tolerant to >2.0	Lawrence, 2007

*Semiquantitative measurement using TetraCare® Easy Strips. Environmental parameters were recorded daily, and the levels of nitrite (mg/L^{-1}), nitrate (mg/L^{-1}) and hardness (mg/L^{-1}) were recorded weekly

Anexo 2.

Primers of target and reference genes used for qRT-PCR.

Target genes	**Gene name**	**# Accession transcripts**	**Forward Primers (5'---3')**	**Reverse primers (5'---3')**	**Amplicon size (bp)**
Myod	Myogenic differentiation 1	ENSDARG00000030110	5'-CAAGAGATGCACGTCCACCA-3'	5'TTCTGAGAAGAGCCTGCAGAGAC-3'	101
Myog	Miogenina	ENSDARG00000009438	5'-CAATGGTGGCTTCGAGCAA-3'	5'-TGTCTTCCAACCCAACTGTGG-3'	101
Mrf4	Myogenic factor 4	ENSDARG00000029830	5'-AAGACGGTGCCTAATCCGAAC-3'	5'-TCCAGCGAATGCAAGAGGTC-3'	101
Igf1a	insulin-like growth factor 1	ENSDARG00000094132	5'-ACGAGCACAACGACACACAGA-3'	5'-TCCTCCCgCTGTCCTCTACA	151
Igf2a	Insulin –like growth factor 2	ENSDARG00000018643	5'-AAGTCGGAGCGAGATGTTTCCT-3'	5'-TCCTTGTGAAGAGCCTGTGACACT-3'	68
mTOR	mechanistic target of rapamycin	ENSDARG00000053196	5'-TCATGCACGACATGAGTGTTGGAC-3'	5'-TCATCCAGGTTAGCTCCGAACAGT-3'	82
Pld1a	phospholipase D1a	ENSDARG00000056228	5'-GGTGCCTTTACTGACCCAAGCATT-3'	5'-CGTCCTTGTAGAAGCTGTTGCTGA-3'	64
Myostatin 1b	myostatin b	ENSDARG00000069133	5'-GCATGTGGTCCAGTGGGTTAT-3'	5'-GTTGTCTGAACTCACATGTGGAACA-3'	100
Reference	**genes**				
Ef1a	Elongation factor 1 alpha	ENSDARG00000020850	5'- TGCCCTTGATGCCATTCTG-3'	5'-TCACAACCATACCAGGCTTG-3'	146
β-actin	Beta actin	ENSDARG00000037870	5'-CGAGCTGTCTTCCCATCCA-3'	5'-CGAGCTGTCTTCCCATCCA-3'	129
Rpl13alfa	Ribosomal protein L13α	ENSDARG00000044093	5'-TCTGGAGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	148

Anexo 3. Mean initial weight (mg) among 24 experimental families at 28 dpf.

Families	N	Mean	
3	26	1.0724	a
7	25	1.1333	ab
4	25	1.1531	ab
2	26	1.1967	abc
22	25	1.2050	abc
1	25	1.2087	abc
23	25	1.2722	abc
11	26	1.3214	abcd
8	25	1.3750	abcd
24	25	1.5125	abcde
14	25	1.5455	abcde
18	25	1.5806	abcde
17	25	1.6313	abcde
21	26	1.6353	abcde
6	25	1.7000	abcde
5	25	1.7000	abcde
9	25	1.7870	bcde
20	26	1.7879	bcde
15	25	1.7900	bcde
10	25	1.8567	cde
13	26	1.9393	de
19	25	2.1485	ef
16	25	2.4304	f
12	26	2.8077	g

Letters indicate the significant differences among families after SNK multiple range test ($P<0.01$).

Anexo 4. Mean weight gain (mg) among 24 experimental families at 70 dpf fed FM (control) and PP (experimental) diets.

	FM diet		PP diet		
Family	N	Mean	N	Mean	
1	14	69.77	15	49.20	**
2	15	48.44	15	35.59	**
3	14	63.43	13	29.17	**
4	14	54.83	15	38.09	**
5	15	79.30	14	52.27	**
6	15	69.17	14	35.11	**
7	14	54.78	15	59.01	**
8	14	69.88	14	42.21	**
9	14	63.23	14	32.75	**
10	15	64.68	14	41.98	**
11	15	76.12	14	41.15	**
12	14	62.69	15	48.59	**
13	15	54.34	14	46.00	**
14	15	85.94	15	45.13	**
15	15	65.43	15	48.13	**
16	15	80.55	14	50.40	**
17	14	63.49	14	43.25	**
18	15	77.52	13	41.25	**
19	15	97.02	14	58.50	**
20	15	66.86	15	39.48	**
21	14	54.18	14	32.90	**
22	15	61.67	14	31.34	**
23	15	61.91	15	38.79	**
24	13	48.09	13	26.78	**
total	349	66.61	342	42.11	**

** indicate significant differences in diet and families (ANOVA, $P<0.01$).

Anexo 5. Mean weight gain (mg) in males among 24 experimental families at 98 dpf fed FM (control) and PP (experimental) diets.

	FM diet Males		PP diet Males		
Family	N	Mean	N	Mean	
1	4	150.89	8	136.01	**
2	10	135.41	9	120.30	**
3	8	175.04	11	129.10	**
4	18	140.14	21	137.10	**
5	10	181.87	12	178.04	**
6	25	156.41	31	110.52	**
7	10	161.86	16	120.76	**
8	9	164.46	13	148.58	**
9	20	154.29	30	120.76	**
10	19	174.22	20	109.85	**
11	5	145.82	8	129.50	**
12	20	158.06	17	147.81	**
13	18	168.36	32	128.77	**
14	18	158.35	34	118.03	**
15	12	96.56	20	91.35	**
16	21	156.48	15	156.38	**
17	7	177.40	11	91.96	**
18	12	178.13	28	132.46	**
19	16	208.95	20	157.95	**
20	6	175.64	15	130.81	**
21	12	140.06	30	120.91	**
22	20	160.90	19	95.57	**
23	18	164.16	16	133.48	**
24	9	146.32	4	147.41	**

** indicate significant differences in diet and families (ANOVA, $P<0.01$).

Anexo 6. Mean weight gain (mg) in females among 24 experimental families at 98 dpf fed FM (control) and PP (experimental) diets.

	FM diet Females		PP diet Females		
Family	N	Mean	N	Mean	
1	34	192.54	23	154.14	**
2	28	178.92	25	118.83	**
3	19	228.52	25	128.21	**
4	21	184.64	15	150.13	**
5	9	289.69	8	212.40	**
6	6	189.28	2	114.45	**
7	15	229.18	11	139.03	**
8	20	207.38	21	140.35	**
9	16	198.99	7	151.65	**
10	15	185.03	18	117.03	**
11	22	188.13	19	167.18	**
12	20	179.37	16	166.46	**
13	17	201.64	6	186.94	**
14	12	230.23	4	135.47	**
15	27	184.92	13	210.24	**
16	12	185.76	7	148.51	**
17	25	189.70	21	175.35	**
18	16	240.57	7	164.24	**
19	11	220.15	5	164.45	**
20	29	190.62	19	159.10	**
21	24	142.78	7	151.80	**
22	14	230.68	17	148.05	**
23	14	226.96	11	147.05	**
24	23	201.90	32	128.51	**
total	449	198.71	339	149.88	**

** indicate significant differences in diet and families (ANOVA, $P<0.01$).

Anexo 7. Fifty four genes over-expressed in lower growth fish

Gen symbol	Description	Ensembl Gene ID	P-value	FDR	Fold Change	RPKM higher growth fish	RPKM lower growth fish	Level expression
BX296557.6	Uncharacterized protein	ENSDARG00000091744	0	0	6.435	319.0435	2053.046	highly
myhz2	myosin, heavy polypeptide 2, fast muscle specific	ENSDARG00000012944	1.64E-04	0.031	5.965	119.6865	713.9455	highly
pvalb2	Parvalbumin-2	ENSDARG00000002768	5.55E-06	1.90E-03	4.727	1065.237	5035.3745	highly
nop10	H/ACA ribonucleoprotein complex subunit 3	ENSDARG00000089426	2.33E-05	6.37E-03	4.648	3.9745	18.473	medium
acta1b	actin, alpha skeletal muscle	ENSDARG00000055618	1.37E-07	7.96E-05	4.007	27.55	110.402	highly
acta1a	actin, alpha 1a, skeletal muscle	ENSDARG00000036371	5.89E-05	0.014	3.738	16.3715	61.2005	medium
scpp6	secretory calcium-binding phosphoprotein 6	ENSDARG00000075598	8.75E-04	0.116	3.721	10.191	37.92	medium
zgc:101853	hypothetical protein LOC494049	ENSDARG00000036894	2.13E-04	0.038	3.719	4.62	17.18	medium
si:dkey-4c2.7	novel protein with a PLAC8 family domain	ENSDARG00000087764	6.56E-04	0.093	3.659	4.533	16.5865	medium
col27a1a	Collagen alpha-1(XXVII) chain A	ENSDARG00000034373	8.33E-04	0.112	3.518	4.219	14.8405	medium
odc1	ornithine decarboxylase	ENSDARG00000007377	1.53E-04	0.029	3.503	5.5245	19.35	medium
PDCL3	phosducin-like 3	ENSDARG00000009449	9.10E-04	0.119	3.319	4.668	15.4945	medium
CABZ01074133.1	CABZ01074133.1	ENSDARG00000078899	9.16E-05	0.021	3.266	6.7745	22.127	medium
actc1b	actin, alpha cardiac muscle 1	ENSDARG00000037840	2.43E-10	1.98E-07	3.025	2545.0815	7698.561	highly
Tnc	tenascin C	ENSDARG00000021948	3.05E-04	0.05	2.985	7.0315	20.993	medium
col12a1	Collagen XII alpha-1 chain	ENSDARG00000078322	8.05E-06	2.58E-03	2.878	11.8295	34.043	medium
phospho1	Probable phosphatase phospho1	ENSDARG00000008403	5.76E-05	0.014	2.853	9.8	27.957	medium
si:ch211-267e7.7	LOC567907 protein	ENSDARG00000021480	1.95E-06	7.70E-04	2.773	14.7105	40.799	medium
actn3a	actinin alpha 3a	ENSDARG00000013755	1.52E-04	0.029	2.756	161.376	444.8315	highly
AL935186.6	Uncharacterized protein	ENSDARG00000088951	2.47E-08	1.60E-05	2.752	1041.7375	2866.745	highly
cct4	T-complex protein 1 subunit delta	ENSDARG00000013475	6.54E-04	0.093	2.618	8.7495	22.908	medium
ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5	ENSDARG00000053481	9.49E-05	0.021	2.558	12.2015	31.2135	medium
Nars	Novel protein similar to vertebrate asparaginyl-tRNA synthetase (NARS)	ENSDARG00000061100	1.12E-05	3.34E-03	2.496	16.4435	41.05	medium
Nefm	neurofilament	ENSDARG00000043697	1.32E-03	0.165	2.495	14.874	37.116	medium

col11a1b	collagen alpha-1(XI) chain	ENSDARG00000009014	3.65E-05	9.37E-03	2.492	14.657	36.519	medium
Fau	40S ribosomal protein S30	ENSDARG00000014830	5.00E-09	3.47E-06	2.485	71.4105	177.4465	highly
CABZ01086825.1	Uncharacterized protein	ENSDARG00000053803	3.17E-06	1.21E-03	2.448	21.0335	51.5	medium
COL12A1	collagen, type XII, alpha 1	ENSDARG00000019601	1.56E-04	0.03	2.356	14.3925	33.9145	medium
wu:fb11d11	hypothetical protein LOC100037361	ENSDARG00000070212	6.54E-05	0.016	2.355	6221.42	14654.299	highly
pdlim7	Pdlim7 protein	ENSDARG00000030638	4.53E-07	2.19E-04	2.349	25.7285	60.4485	medium
si:dkey-32e6.6	Novel protein similar to vertebrate extracellular matrix protein 2, female organ and adipocyte specific	ENSDARG00000070857	7.98E-07	3.55E-04	2.343	36.7855	86.1735	medium
rpl18a	60S ribosomal protein L18a	ENSDARG00000025073	1.40E-13	2.21E-10	2.328	118.8545	276.683	highly
rpl15	60S ribosomal protein L15	ENSDARG00000009285	1.33E-14	2.71E-11	2.325	61.8565	143.789	highly
col11a2	collagen alpha-2(XI) chain	ENSDARG00000012422	1.53E-05	4.35E-03	2.324	19.573	45.4905	medium
si:ch73-92o9.2	novel transcript	ENSDARG00000061081	5.70E-04	0.084	2.321	12.4715	28.946	medium
Mdka	midkine-related growth factor	ENSDARG00000036036	1.02E-03	0.131	2.305	18.7905	43.317	medium
dnajc8	dnaJ homolog subfamily C member 8	ENSDARG00000059373	2.69E-04	0.045	2.29	14.5535	33.323	medium
rps6	40S ribosomal protein S6	ENSDARG00000019778	1.91E-06	7.65E-04	2.274	25.267	57.465	medium
zgc:158399	hypothetical protein LOC570789	ENSDARG00000060065	4.98E-04	0.075	2.269	34.9975	79.412	medium
ryr3	Ryanodine receptor 3	ENSDARG00000071331	7.06E-06	2.34E-03	2.255	126.872	286.128	highly
rpl10	60S ribosomal protein L10	ENSDARG00000025581	8.06E-06	2.58E-03	2.217	32.3075	71.6205	medium
zgc:77231	fast skeletal myosin alkali light chain 1	ENSDARG00000014196	4.85E-11	4.45E-08	2.212	3196.258	7069.485	highly
zgc:165344	myosin-binding protein H-like	ENSDARG00000003081	5.78E-05	0.014	2.195	251.8665	552.84	highly
nap1l1	nucleosome assembly protein 1-like 1	ENSDARG00000002400	0	0	2.169	136.967	297.136	highly
rpl13	60S ribosomal protein L13	ENSDARG00000030585	2.05E-05	5.68E-03	2.162	98.876	213.7825	highly
rpl6	60S ribosomal protein L6	ENSDARG00000058451	7.52E-06	2.46E-03	2.156	193.475	417.2035	highly
Ppia	2-peptidylprolyl isomerase A	ENSDARG00000009212	2.90E-07	1.49E-04	2.14	35.3245	75.6115	medium
Eprs	LOC562037 protein	ENSDARG00000060494	2.89E-04	0.048	2.134	17.881	38.161	medium
rps29	40S ribosomal protein S29	ENSDARG00000041232	7.56E-09	5.13E-06	2.132	122.182	260.4375	highly
Postn	periostin, osteoblast specific factor	ENSDARG00000043806	1.09E-05	3.29E-03	2.125	28.8335	61.2645	medium
Ucma	upper zone of growth plate	ENSDARG00000027799	0	0	2.101	115.223	242.0425	highly

klhl31	Kelch-like protein 31	ENSDARG00000039066	1.57E-03	0.187	2.073	19.234	39.8695	medium
eef2b	eukaryotic translation elongation factor 2b	ENSDARG00000003016	2.79E-04	0.046	2.067	88.127	182.134	highly
gnb2l1	Guanine nucleotide-binding protein subunit beta-2-like 1	ENSDARG00000041619	5.78E-04	0.085	2.04	18.7945	38.338	medium

Anexo 8. Seventy genes over-expressed gene in higher growth fish

Gene symbol	Description	Ensembl Gene ID	P-value	FDR	Fold Change	RPKM in higher growth	RPKM in lower growth	level of expression
Ebp	emopamil binding protein (sterol isomerase)	ENSDARG00000046098	6.30E-04	0.091	-24.453	58.0705	2.375	medium
sc5dl	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)	ENSDARG00000044642	1.08E-04	0.023	-14.38	20.149	1.4015	medium
sc4mol	sterol-C4-methyl oxidase	ENSDARG00000055876	1.60E-05	4.52E-03	-14.123	111.416	7.889	highly
zgc:92111	novel protein	ENSDARG00000045306	6.53E-04	0.093	-12.447	113.3875	9.1095	highly
dhcr24	24-dehydrocholesterol reductase	ENSDARG00000013236	3.35E-05	8.69E-03	-10.389	29.6225	2.8515	medium
CABZ01071923.1	Uncharacterized protein	ENSDARG00000089599	5.20E-04	0.078	-10.278	85.242	8.2935	medium
Nsdhl	NAD(P) dependent steroid dehydrogenase	ENSDARG00000057723	8.75E-04	0.116	-10.04	8.1275	0.8095	low
HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	ENSDARG00000091751	4.59E-04	0.07	-9.126	26.294	2.8815	medium
dhcr7	7-dehydrocholesterol reductase	ENSDARG00000015564	5.89E-07	2.75E-04	-8.938	22.1465	2.478	medium
zgc:92880	zgc:92880	ENSDARG00000069734	1.13E-03	0.143	-8.26	7.5075	0.909	low
hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	ENSDARG00000052738	2.65E-14	5.03E-11	-7.29	44.2095	6.0645	medium
Fdps	farnesyl diphosphate synthase	ENSDARG00000040890	1.31E-04	0.026	-7.259	43.055	5.9315	medium
acat2	acetyl-CoA acetyltransferase 2	ENSDARG00000007127	2.46E-04	0.042	-7.184	13.3715	1.8615	medium
si:dkey-97i18.5	novel transcript	ENSDARG00000090352	3.78E-14	6.33E-11	-6.899	43.73	6.3385	medium
cyp51	cytochrome P450, family 51	ENSDARG00000042641	8.74E-06	2.71E-03	-6.729	15.561	2.3125	medium
SQLE	squalene epoxidase	ENSDARG00000079946	1.53E-03	0.184	-5.513	13.619	2.4705	medium
entpd8	ectonucleoside triphosphate diphosphohydrolase 8	ENSDARG00000005565	2.62E-12	3.24E-09	-5.51	41.9295	7.6095	medium
si:dkey-266f7.7	novel protein similar to envelope protein	ENSDARG00000009890	3.35E-11	3.25E-08	-5.466	285.177	52.173	highly
CABZ01060296.1	Uncharacterized protein	ENSDARG00000090867	1.62E-03	0.189	-5.311	8.6975	1.638	low
lmod2b	leiomodin 2 (cardiac) b	ENSDARG00000045864	1.08E-05	3.29E-03	-4.968	17.579	3.5385	medium
Muc17	mucin 17, cell surface associated	ENSDARG00000075038	3.44E-04	0.054	-4.57	12.3125	2.6945	medium

sult2st3	sulfotransferase family 2, cytosolic sulfotransferase 3	ENSDARG00000028367	9.18E-07	3.92E-04	-4.408	66.327	15.047	medium
C3H17orf37	chromosome 17 open reading frame 37	ENSDARG00000089936	2.54E-06	9.92E-04	-4.307	37.7045	8.755	medium
CABZ01111729.1	CABZ01079804.1	ENSDARG00000059061	9.72E-05	0.021	-4.295	29.3635	6.8365	medium
FDFT1	farnesyl-diphosphate farnesyltransferase 1	ENSDARG00000060260	8.52E-04	0.114	-4.065	11.649	2.866	medium
cyp7a1a	cytochrome P450, family 7, subfamily A, polypeptide 1a	ENSDARG00000069018	1.30E-04	0.026	-3.67	17.695	4.822	medium
CR854881.1	Uncharacterized protein	ENSDARG00000058556	1.37E-03	0.169	-3.645	11.7585	3.226	medium
hsd3b7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase	ENSDARG00000036966	5.24E-13	7.47E-10	-3.611	60.5115	16.758	medium
VDAC3	voltage-dependent anion channel 3	ENSDARG00000073775	3.23E-04	0.052	-3.513	15.378	4.377	medium
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	ENSDARG00000077402	4.45E-07	2.19E-04	-3.477	30.6085	8.8035	medium
si:dkey-91i10.3	novel protein similar to vertebrate cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1)	ENSDARG00000057262	2.73E-04	0.046	-3.39	16.345	4.822	medium
AGPHD1	aminoglycoside phosphotransferase domain containing 1	ENSDARG00000074341	1.51E-03	0.182	-3.32	28.1115	8.4675	medium
CR385063.1	Uncharacterized protein	ENSDARG00000044212	2.32E-07	1.27E-04	-3.129	35.8395	11.4545	medium
fdx1	ferredoxin 1	ENSDARG00000056410	2.08E-11	2.19E-08	-3.121	60.3555	19.3385	medium
cyb5b	cytochrome b5 type B	ENSDARG00000053954	7.00E-04	0.097	-3.112	24.6795	7.9315	medium
idh1	isocitrate dehydrogenase 1 (NADP+), soluble	ENSDARG00000025375	5.90E-05	0.014	-3.088	21.932	7.102	medium
Hmgcra	3-hydroxy-3-methylglutaryl-Coenzyme A reductase a	ENSDARG00000052734	1.14E-04	0.023	-3.039	20.6385	6.7925	medium
ATPIF1	ATPase inhibitory factor 1	ENSDARG00000044092	3.22E-04	0.052	-2.957	775.4305	262.243	highly
PHLDB3	pleckstrin homology-like domain, family B, member 3	ENSDARG00000091016	1.47E-03	0.178	-2.899	14.83	5.115	medium
atp5ia	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e, duplicate a	ENSDARG00000078113	1.60E-03	0.189	-2.881	203.378	70.586	highly
chchd10	coiled-coil-helix-coiled-coil-helix domain containing 10	ENSDARG00000010717	6.72E-04	0.095	-2.78	28.962	10.417	medium
CABZ01079804.1	CABZ01079804.1	ENSDARG00000088988	2.49E-11	2.54E-08	-2.756	287.928	104.469	highly

acaa2	acetyl-CoA acyltransferase 2	ENSDARG00000038881	6.92E-04	0.097	-2.736	18.1725	6.6425	medium
mt-cyb	cytochrome b, mitochondrial	ENSDARG00000063924	1.16E-23	3.67E-20	-2.722	10388.869	3816.8965	highly
papss2a	3'-phosphoadenosine 5'-phosphosulfate synthase 2a	ENSDARG00000071021	7.83E-04	0.107	-2.714	18.031	6.6425	medium
reep3	receptor accessory protein 3	ENSDARG00000004160	3.39E-04	0.054	-2.69	20.729	7.705	medium
BX927387.2	Uncharacterized protein	ENSDARG00000073764	7.46E-04	0.103	-2.673	18.556	6.9415	medium
smtnl1	smoothelin-like 1	ENSDARG00000041257	3.68E-14	6.33E-11	-2.658	94.231	35.457	medium
baiap2l1a	BAI1-associated protein 2-like 1a	ENSDARG00000029305	3.02E-08	1.87E-05	-2.61	51.7035	19.808	medium
mt-nd4	NADH dehydrogenase 4, mitochondrial	ENSDARG00000063917	1.75E-05	4.87E-03	-2.587	5922.8025	2289.531	highly
CT027607.3	fatty acid binding protein 11a	ENSDARG00000017299	4.26E-04	0.065	-2.583	88.746	34.361	medium
brp44	brain protein 44	ENSDARG00000024478	1.20E-05	3.53E-03	-2.478	34.7055	14.0035	medium
ARHGEF5	Rho guanine nucleotide exchange factor (GEF) 5	ENSDARG00000076383	8.97E-05	0.021	-2.461	67.313	27.353	medium
tnni1al	troponin I, skeletal, slow like	ENSDARG00000036671	3.55E-30	1.68E-26	-2.364	303.8725	128.563	highly
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	ENSDARG00000036329	7.39E-12	8.77E-09	-2.314	143.009	61.81	highly
slc25a14	solute carrier family 25 (mitochondrial carrier, brain), member 14	ENSDARG00000026680	4.11E-05	0.01	-2.277	34.9205	15.3365	medium
ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10	ENSDARG00000013333	8.71E-06	2.71E-03	-2.276	41.2915	18.145	medium
zgc:153662	novel protein	ENSDARG00000042559	1.43E-26	5.10E-23	-2.273	429.501	188.9175	highly
mt-nd1	NADH dehydrogenase 1, mitochondrial	ENSDARG00000063895	2.94E-07	1.49E-04	-2.227	3830.5625	1719.8445	highly
aco2	aconitase 2, mitochondrial	ENSDARG00000007294	1.76E-18	4.57E-15	-2.189	192.7445	88.066	highly
mt-nd5	NADH dehydrogenase 5, mitochondrial	ENSDARG00000063921	8.62E-11	7.45E-08	-2.159	2273.4565	1052.915	highly
CKMT2	creatine kinase, mitochondrial 2 (sarcomeric)	ENSDARG00000069615	6.47E-21	1.84E-17	-2.157	333.244	154.5085	highly
uqcrc1	ubiquinol-cytochrome c reductase core protein I	ENSDARG00000052304	1.10E-11	1.26E-08	-2.15	106.777	49.6745	highly
ADCK3	aarF domain containing kinase 3	ENSDARG00000020123	5.90E-06	2.00E-03	-2.136	63.923	29.92	medium
Glulb	glutamate-ammonia ligase (glutamine synthase) b	ENSDARG00000017339	4.75E-06	1.67E-03	-2.12	59.621	28.1195	medium
mt-co2	cytochrome c oxidase II, mitochondrial	ENSDARG00000063908	1.46E-11	1.60E-08	-2.105	5281.0855	2508.3265	highly

COX6B1	cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	ENSDARG00000037860	3.42E-11	3.25E-08	-2.038	737.002	361.657	highly
mt-co1	cytochrome c oxidase I, mitochondrial	ENSDARG00000063905	9.51E-04	0.123	-2.029	19352.1265	9537.9745	highly
ndufb10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	ENSDARG00000028889	1.92E-07	1.09E-04	-2.012	84.536	42.025	medium
mt-nd2	NADH dehydrogenase 2, mitochondrial	ENSDARG00000063899	3.59E-06	1.31E-03	-2.008	2713.1745	1351.05	highly

Rev Fish Biol Fisheries
DOI 10.1007/s11160-011-9203-0

REVIEWS

Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes

Pilar E. Ulloa · Patricia Iturra · Roberto Neira · Cristian Araneda

Received: 29 August 2010 / Accepted: 14 February 2011

Abstract Zebrafish (*Danio rerio*) is a common research model in fish studies of toxicology, developmental biology, neurobiology and molecular genetics; it has been proposed as a possible model organism for nutrition and growth studies in fish. The advantages of working with zebrafish in these areas are their small size, short generation time (12–14 weeks) and their capacity to produce numerous eggs (100–200 eggs/clutch). Since a wide variety of molecular tools and information are available for genomic analysis, zebrafish has also been proposed as a model for nutritional genomic studies in fish. The detailed study of every species employed as a model organism is important because these species are used to generalize how several biological processes occur in related organisms, and contribute considerably toward improving our understanding of the mechanisms involved in nutrition and growth. The objective of this review is to show the relevant aspects of the nutrition and growth in zebrafish that support its utility as a model organism for nutritional genomics studies. We made a particular emphasis that gene expression and genetic variants in response to zebrafish nutrition will shed light on similar processes in aquacultured fish.

Keywords Zebrafish · Growth · Nutrition · Nutritional genomics · Comparative genomics

P. E. Ulloa (✉)
Departamento de Ciencias Químicas, Programa de Doctorado en Ciencias de Recursos Naturales, Facultad de Ciencias Ingeniería y Administración, Universidad de La Frontera, Casilla 54-D, Temuco, Chile
e-mail: pilarelizabeth@gmail.com

P. Iturra
Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 1004, Santiago, Chile

P. E. Ulloa · R. Neira · C. Araneda
Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 70061-7, Santiago, Chile

R. Neira
AQUAINNOVO S.A., Polpaico 037, Puerto Montt, Chile

Introduction

The zebrafish has become a very common research model in development, neurobiology and molecular genetics (Driever et al. 1994; Roush 1996; Bergeron et al. 2008). Zebrafish have recently been proposed as a possible model organism for nutrition and growth studies in fishes (Alestrom et al. 2006; Dahm and Geisler 2006; De-Santis and Jerry 2007; Wright et al. 2006; Johnston et al. 2008).

Growth is a trait of main interest because it is closely linked with the productivity and profitability of aquaculture production (De-Santis and Jerry 2007). Phenotypic expression of this trait is under genetic control, but also depends upon environmental effects,

Published online: 26 February 2011

and is directly affected by nutrition (Moriyama et al. 2000). Advances in genetic technologies have opened a window to understand the genetic variation underlying quantitative traits, among which the identification of candidate genes for growth and development is remarkable, along with the mapping of genomic regions of quantitative trait loci (QTL) which affect growth traits in cultured species (Davis and Hetzel 2000; Fjalestad et al. 2003; Reid et al. 2005; Araneda et al. 2008; Lo Pestri et al. 2009; Dumas et al. 2010). In this aspect, the zebrafish has a wider variety of molecular tools and information available for genomic analysis than any other cultured species.

The zebrafish has been proposed as a model fish for nutritional genomic studies, with the expectation that results from this approach will provide comparative genomic information applicable to aquacultured fish (Metscher and Ahlberg 1999; Drew et al. 2008; Robison et al. 2008; Crollius and Weissenbach 2008). Nutritional genomics is a discipline which integrates nutrition and genetics. Is a discipline that investigates gene-nutrient interactions using two approaches: "Nutrigenomics", which studies how diet affects the expression of certain genes, and "Nutrigenetics", which studies the genetic variants that influence the organism's response to nutrients (Kaput et al. 2003; Müller and Kersten 2003; Mutch et al. 2005; Marti et al. 2005; Panserat et al. 2007). In aquacultured fish some studies have been developed in nutrigenomics during the fasted-fed transition, as well as after the replacement of dietary fish meal or fish oil by plant ingredients (Panserat and Kaushik 2010). Investigating plant food sources became important due to the change of fish diets in the aquafeed sector, where plant derived ingredients are increasingly used due to the global demand of fishmeal (Hardy 2010; Turchini et al. 2009). These nutrigenomic studies help us to understand how a change in the formulation of the diet produces multiple physiological responses in fish at the trasncriptional level.

Nutrigenetics refers to the analysis of specific genetic differences between individuals that respond in different ways to the same diet. These individual variations often occur as single nucleotide polymorphisms (SNPs) in coding genes, which may influence the expression of a particular phenotype (Vignal et al. 2002; Liu and Cordes 2004). SNPs are point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. SNPs are becoming a focal point in molecular marker development since they are the most abundant polymorphisms. In zebrafish there is one SNP for every 145–219 bp. They have a codominant inheritance pattern, are adaptable to automated genotyping, and reveal hidden polymorphisms not detected with other markers and methods (Stickney et al. 2002; Vignal et al. 2002; Liu and Cordes 2004). SNP variants may be responsible for the phenotypic variation of some important aquaculture quantitative traits such as growth related traits. Identification of functional polymorphisms in zebrafish is a step toward SNP association with productive traits (Fjalestad et al. 2003; Ryynänen and Craig 2006; Lo Pestri et al. 2009; De-Santis and Jerry 2007). The main objective of this review is to present the most relevant aspects of the nutrition and growth in zebrafish that supports its utility as a model organism for nutritional genomics studies in aquacultured fish with special utility to salmonids.

Advantages of zebrafish as a model organism

Zebrafish have advantages biology as a model organism that include their small size, short generation time interval (12–14 weeks), their capacity to produce numerous offspring, breed easily and very amenable to manipulation in a laboratory tank (Clark 2003). Moreover, zebrafish have a wide variety of molecular tools and information available for genomic analysis, and a characterized genome compared to aquacultured fishes. The eighth revision (Zv8) of sequenced genome of the zebrafish was concluded in 2008 (Orban and Wu 2008) and recently in October 2010 the Zebrafish Genome Project with to the Genome Reference Consortium at NCBI (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/index.shtml) completed a new revision (Zv9).

The existence of shared synteny between human and zebrafish genomes has been demonstrated (Barbazuk et al. 2000), as well as between zebrafish and green spotted pufferfish (*Tetraodon nigriviridis*) (Woods et al. 2005). Evidence obtained from rainbow trout (*Oncorhynchus mykiss*) genetic maps also show synteny between zebrafish and trout (Rexroad et al. 2005, 2008). Considering the synteny between genomes of different species and the existing evidence, recent revisions concluded that there should be

abundant synteny between the zebrafish and salmonid genomes at the level of small regions that include conserved genes. In a sequencing study of ~1 Mb of Atlantic salmon (*Salmo salar*), gene order in a region was conserved in three model fish species medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and zebrafish, but only in zebrafish was it possible to identify similar genes to salmon (Quinn et al. 2008). The generation of 43,000 BAC end sequences (BES) reported for catfish shows conserved syntenic regions between catfish (*Ictalurus punctatus*) and zebrafish genome. A total of 10,943 catfish BES (17.3%) had significant BLAST hits to the zebrafish genome, with 3,221 unique gene hits, providing a platform for comparative mapping based on locations of these catfish genes in the zebrafish genome (Liu et al. 2009). Thus, given the synteny of these genomes, it is possible to use the genomic resources that zebrafish possess and adapt them for use in fish species of economic importance.

Genomic and genetic resources of zebrafish and other aquacultured fishes

The zebrafish genome has an average C-value of 1,848 pg (Animal Genome Size Database) and is estimated to contain 1.7×10^9 base pairs of DNA which is about half the size of the mammalian genome (Postlethwait 2004). Zebrafish information available includes 35,204 genes; 1,481,937 ESTs (expressed sequence tags); 161,330 GSS (genome survey sequence) and 662,336 SNPs (National Center for Biotechnology Information; NCBI). In zebrafish 2,035 SNPs in 712 genes were identified by Stickney et al. (2002) and later Woods et al. (2005) developed a more complete map with 4,073 mostly SNP markers in genes and ESTs. Bradley et al. (2007) identified approximately 550,000 potential SNPs in 39% of the genomic sequence of zebrafish. They verified subgroups of these SNPs, found that over 70% may be validated polymorphisms and estimated that near 390,000 corresponded to available SNPs. Up to the present, SNP application in teleosts has been restricted to salmonids, using conserved sites (synteny) to validate SNPs between species (Smith et al. 2005).

On the other hand, a rapid development of genetic and genomics resources has been made recently for economically important aquacultured fish species such as channel catfish (Li et al. 2007; Xu et al. 2007) rainbow trout (Rexroad III et al. 2003; Govoroun et al. 2006), Atlantic salmon (Thorsen et al. 2005; Davidson et al. 2010), Atlantic cod (*Gadus morhua*) (Nielsen et al. 2006; Wesmajervi et al. 2007; Johansen et al. 2009) and Gilthead sea bream (*Sparus aurata*) (Franch et al. 2006; Senger et al. 2006; Sarropoulou et al.2007).

Genomic information for Atlantic salmon includes 3,974 genes; 498,212 ESTs; 203,387 GSS and 1,344 SNP (NCBI, January 2011). Recently 9,057 full-length reference genes were characterized in this salmon (Leong et al. 2010), and several SNP markers have been discovered. For example, 2,507 putative SNPs from the alignment of 100,866 ESTs were discovered by Hayes et al. (2007). In the same way Boulding et al. (2008) identified 129 SNPs within ESTs spaced throughout the linkage map and identified 79 SNPs markers on linkage groups associated with QTLs for growth rate, condition factor, shape and skin pigmentation traits. Moen et al. (2008a) developed 1,369 SNPs markers of which 304 are located within genes anchored in a linkage map. A panel of 15,225 SNPs from the Atlantic salmon SNP chip (Center for Integrative Genetics, CIGENE) was evaluated as a potential genomic tool for whole genome selection (WGS), where 2,991 single copy polymorphic SNPs were considered useful for further analysis (Dominik et al. 2010).

Genetic resources for catfish include over 500,000 ESTs (Wang et al. 2010; Li et al. 2007), over 10,000 full-length cDNA (Chen et al. 2010), over 300,000 putative SNPs (Wang et al. 2010), and the development of high-density SNP chip is in progress (Lu et al. 2010). In Atlantic Cod 724 putative SNP developed from 17,056 EST were evaluated, from which 318 segregating SNPs were selected for future genetic analysis (Moen et al. 2008b). In sea bream a long-read EST database was generated, containing 1,393 cDNA clones representing 852 unique cDNA sequence-reads (Sarropoulou et al. 2005). Seventy-six SNPs markers for use in studies of natural populations and selective breeding were validated in this species (Cenadelli et al. 2007). The large numbers of SNP discoveries in different species represent a valuable genetic resource and may be used for performing genome scans of genes or of quantitative trait loci (QTL) influencing

commercially important traits by comparative genomic analysis.

The most powerful genomic resource in zebrafish is the accessibility of its complete genome sequence (Grunwald and Eisen 2002; Orban and Wu 2008). An International Collaboration by Canada, Chile and Norway is being undertaken to obtain a similary complete sequence of the genome of Atlantic salmon (Davidson et al. 2010). Also, efforts to sequence the complete genome of Atlantic cod and catfish has been initiated and is currently in progress (Johansen et al. 2009; Lu et al. 2010). Given that these genome sequencing projects from different aquacultured fishes are underway, the research community can find available information from diverse data sources (Table 1). Fish Map is a unified and centralized resource for storage, retrieval, and display of genomic information on zebrafish. FishMap is built on the Gbrowse, and is a part of the Generic Model Organism Database Consortium Project (GMOD) (Meli et al. 2008). cBARBEL (Catfish Breeder and Researched Bioinformatics Entry Location) is an important catfish genome database and represents one of the first comprehensive bioinformatic databases for an aquaculture species (Lu et al. 2010). Salmon DB a new Chilean multi-organism public access database (on registration) has been recently created. It contains EST sequences from Atlantic salmon, rainbow trout and whole genome sequences from five model fish species (zebrafish, medaka, pufferfish, fugu and stickleback). It was built using the core components from the Generic Model Organism Database (GMOD) project and the Gene Ontology and Pathways Architecture (GOPArc) system (Table 1).

Relationships of zebrafish and other fish models

Five teleosteos fish genomes have been fully sequenced including zebrafish (from Cypriniformes), Japanese fugu (from Tetraodontiformes), green spotted pufferfish (from Tetraodontiformes), medaka (from Beloniformes), and three-spined stickleback (*Gasterosteus aculeatus*, from Gasterosteiformes). Fugu and pufferfish have been sequenced by whole genome shotgun (WGS) assembly (Aparicio et al. 2002). They have very small genome sizes, low nuclear DNA content, less than 500 million base pairs (Mb) per haploid genome (Aparicio et al. 2002; Clark 2003), and have been selected as a model for the human genome project (Cossins and Crawford 2005). However, both fugu and tetraodon will only be used as model genomes, but not as model organism because they do not breed easily in captivity (Westerfield 2000; Lawrence 2007; Clark 2003). Stickleback is a model fish used to addres questions related to adaptation, speciation, and evolution (Bell 2001; Cresko et al. 2007). It is a small fish (adult total body length <10 cm), survives and breeds in both seawater and freshwater; displays a variety of pronounced reproductive behaviors; has a simple and short life cycle of 1 year in nature, but can be brought to sexual maturity in the laboratory within 7–8 months after hatching (Hahlbeck et al. 2004; Katsiadaki et al. 2002).

Medaka is another model fish with a genome size estimated at 650–1,000 Mb, its genome is one-third the size of the human genome and is less than half the size of the zebrafish genome (Hinegardner and Rosen 1972; Kasahara et al. 2007, Clark 2003). Medaka, tolerate a wide range of salinities and temperatures (10–40°C), are small (3–4 cm), have eggs transparent, are easy to breed and with a generation interval time of 6–8 weeks (Wittbrodt et al. 2002). Medaka and zebrafish phylogenetically are separated from their last common ancestor $\sim$110 million years (Myr) ago (Fig. 1). This evolutionary distance is reflected in many aspect of their biology, including their early development. With an evolutionary distance of $\sim$60 Myr (compared with 110–160 Myr in zebrafish), medaka is a much closer relative to fugu and stickleback than it is to zebrafish (Fig. 1), while zebrafish represent a more ancestral condition.

Zebrafish have a much to offer as a model fish in nutrition genomic studies, but they certainly are not perfect. In most vertebrates growth is determined, which means that growth ends at a given moment in the life of the animal and they reaches a finite size. In contrast, fish muscle growth is not lineal and occurs through a combination of recruitment of new muscle fibers (hyperplasia) and an increase of the size of existents fibers (hypertrophy) in post-juvenile stages (Johnston 1999; Mommsen 2001). In contrast to other fishes, zebrafish have determinate growth, the number of muscle fibers is established and fixed at the time of the birth; and growth is solely due to hypertrophy of fibers already formed (Du 2004). This condition does not provide a general model for muscle growth since

Table 1 The main web-based data sites currently available for zebrafish and aquacultured fishes (according to Briggs 2002; Clark 2003; Meli et al. 2008; Johansen et al. 2009; Lu et al. 2010)

Specie	Website name	Internet link
Zebrafish (*Danio rerio*)	Zfin	http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg
	NCBI Zebrafish Genome Resources	http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/index.html
	Entrez Map Viewer	http://www.ncbi.nlm.nih.gov/projects/mapview/static/MapViewerHelp.html
	Ensembl genome browser	http://www.ensembl.org/Danio_rerio/Info/Index
	Zebrafish Mutation Resource	http://www.sanger.ac.uk/Projects/D_rerio/mutres/
	DFCI Zebrafish Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=zfish
	FishMap	http://fishmap.igib.res.in
Rainbow trout (*Oncorhynchus mykiss*)	Rainbow Trout Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=r_trout
	NAGRP Aquaculture Genome Projects	http://www.animalgenome.org/cgi-bin/host/rainbow/viewmap
	Sigenae Contig Browser	http://public-contigbrowser.sigenae.org:9090/Oncorhynchus_mykiss/index.html
Atlantic Salmon (*Salmo salar*)	Atlantic Salmon Gene Index	http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=salmon
	cGRASP consortium	http://web.uvic.ca/cbr/grasp
	SalmoDB	http://genomicasalmones.dim.uchile.cl/
	Asalbase	http://www.asalbase.org/sal-bin/index
Catfish (*Ictalurus* spp.)	cBARBEL	http://catfishgenome.org
Atlantic Cod (*Gadus morhua*)	Atlantic Cod Genomics and Broodstock Development	http://www.codgene.ca
	The Cod Genome Project	http://www.codgenome.no

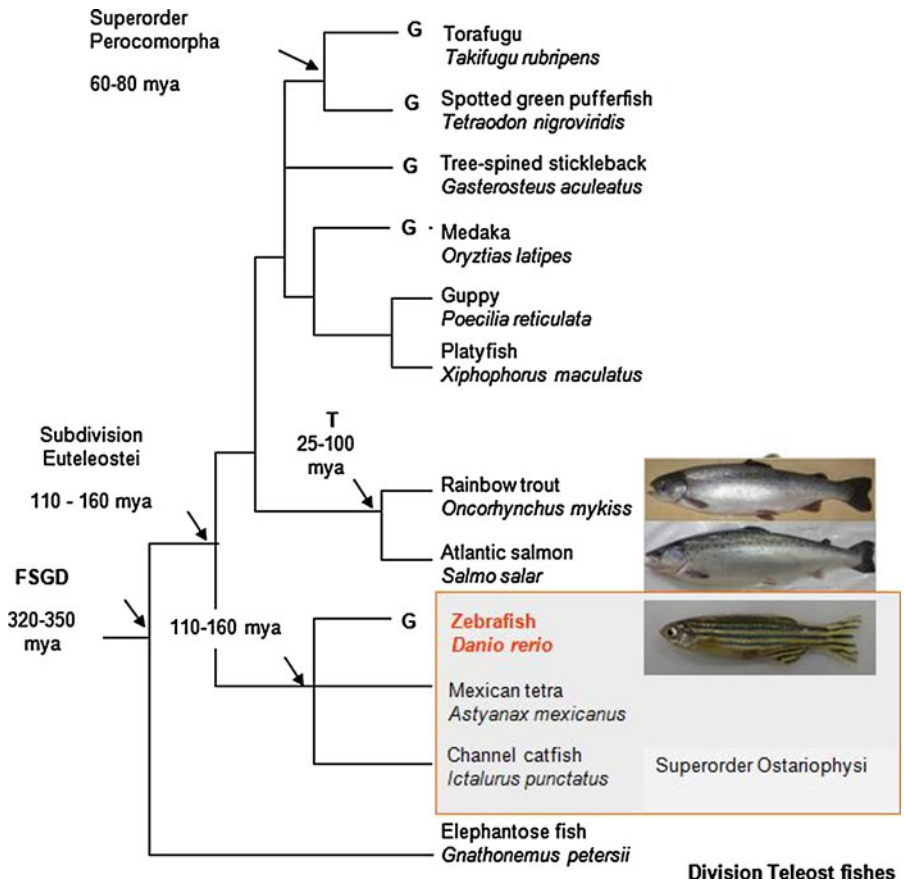

Fig. 1 Teleost relationships of the most commonly studied teleosts. This genealogical tree illustrates fish-specific genome duplication (FSGD), salmonid-specific tetraploidization (T) and fish genome assembly (G). Notably, zebrafish represents a more ancestral condition compared to other model organisms as such medaka. These conditions enabling some translational biological processes addressed more effectively in zebrafish (Adapted from Wittbrodt et al. 2002; Volff 2005)

it only reaches a modest ultimate body size (3–5 cm) (Johnston 1999; Mommsen 2001). However muscle differentiation, somite formation and subsequent differentiation of myoblasts are under the control of complex signaling pathways and of myogenic regulatory factors that are similar in all teleosteos (Du 2004). Understanding how these signaling cascades coordinate with others and how they control myogenic transcriptional networks which change gene expression during muscle differentiation may be unraveled through nutrigenomic studies in zebrafish.

Phylogenetic relationships of zebrafish and aquaculture fishes species

The last common ancestor of all the vertebrate laboratory models was the common ancestor of the Actinopterygii (ray-finned fishes) and Sarcopterygii (lobe-finned fishes and tetrapods), which probably lived during the Silurian period, approximately 420 million years ago (Janvier 1996; Hedges 2002; Clark 2003). Within the ray-finned fishes the dominant group today is the Teleosts which has over 20,000 species, including important aquaculture species such as the Atlantic salmon, rainbow trout, sea bass, and carp (*Cyprinus carpio*) (Clark 2003). The teleosts began a major evolutionary radiation in the Triassic, about 200 million years ago, and have since undergone massive diversification in morphology, physiology and habitat. Their genomes did not remain static and they are still evolving. Their evolutionary divergence and extreme diversity provide an abundant source of different genomes with all the structure–function combinations that have survived during the last 400 million years (Volff 2005). The class Actinopterygii includes those taxa considered as the most ancient fishes (Lauder and Liem 1989). As a group, they are characterized by many derived characteristics which are absent in primitive ray-fins. Teleosts are thus morphologically remote from the common actinopterygian/sarcopterygian ancestor.

However, within the Teleosts, the Ostariophysi (and thus zebrafish) retain many primitive characteristic and occupy a relatively basal position (Lauder and Liem 1989). Thus the zebrafish is a rather generalized teleost and can, in most cases, be used to represent the "primitive" or "ancestral" condition in comparison with more modern laboratory teleosts such as the medaka and fugu (Lauder and Liem 1989; Metscher and Ahlberg 1999) (Fig. 1). With an evolutionary separation of less than 150 million years, the zebrafish is still closer to the aquacultural fish species than any mammalian model organism such as a mouse, whose common ancestor with the teleosts lived around 400 million years ago (Dahm and Geisler 2006). This evolutionary proximity between zebrafish and aquacultured fish species is reflected in many aspects of their biological similarity, and offers several advantages combining the power of genetics with experimental embryology, molecular biology and genomic resources (Wittbrodt et al. 2002).

The plasticity of fish genomes could be manifested by the size of their genome and chromosome number. The haploid zebrafish genome has 25 chromosomes which is similar of the karyotype of the original teleost (Ojima 1983; Daga et al. 1996; Gornung et al. 1997) after an event of fish genomic duplication ($n = 24$; Woods et al. 2005). Most teleosts have around 24–25 haploid chromosomes with very little variation, except in the case of certain lineages such as the salmonids, which contain about 30–40 haploid chromosomes (Allendorf and Thorgaard 1984; Clark 2003; Naruse et al. 2004). Chromosome number distribution in teleosts is quite restricted, in contrast to the broadly distributed chromosome number observed in tetrapodes (Naruse et al. 2004). Comparative genome analysis of medaka and zebrafish with reference to the human gene map indicates that karyotype evolution in teleosts occurred by chromosome fragmentation; mainly inversions (intra-chromosomal rearrangements) and by translocations (inter-chromosomal rearrangements), but not by fusion and/or fission of chromosomes as is observed in mammalian chromosome evolution (Ehrlich et al. 1997; Naruse et al. 2004). Taken together these findings, suggest that teleost karyotypes and the gene content of individual chromosomes should be more similar to the karyotype of the last common ancestor of ray-fin and lobe-fin fish than that of most mammalian genomes (Postlethwait 2004; Woods et al. 2005).

Life cycle of the Zebrafish

In typical laboratory conditions (28.5°C and 14:10 light–dark hours), zebrafish develop very rapidly. Embryogenesis takes 24 h post fertilization (hpf) (Dahm 2002). Larvae hatch within 2.5–3 days post-fertilization (dpf) and the transition from larva to juvenile (metamorphosis) occurs at $\sim$30 dpf (Kimmel et al. 1995). Zebrafish reach sexual maturity from 10 to 14 weeks post-fertilization (Eaton and Farley 1974a). Under favorable conditions zebrafish spawn continuously after sexual maturation (Eaton and Farley 1974b; Breder and Rosen 1966). Their high reproductive capacity and their short generation time (12–14 weeks) serve as an advantage over cultured fishes that present a long generation interval (for example in salmonids this generation interval is 2–4 years, depending upon the species) (Wegrzyn and Ortubay 2009). In a typical domestic salmonid such as the anadromous rainbow trout, once fertilization occurs the incubation period depends essentially on temperature, requiring about 30 days at 10°C or 300 accumulated thermal units (ATU) to reach 50% hatching (Estay et al. 1994). Juvenile fish or "parr", identifiable by their vertical and black spots on both sides of the body (with 1,200 ATU; 3–15 g weight), remain 1 year in freshwater until smoltification (a metabolic processes which adapts a fish from freshwater to marine water); smolt (with 3,200 ATU; 80–120 g) spend another year in marine water until harvest (3–4.5 kg). Depending on the quality of food and genetic factors, sexual maturity is reached in males in the second or third year of life and in females in the third or fourth year (Wegrzyn and Ortubay 2009). These conditions: long generation interval and late sexual maturity, limit population level studies. A comparative view of zebrafish and rainbow trout life cycles, giving the time periods for their different stages of life, is shown in Fig. 2.

Digestive system of adult zebrafish

Fish have different feeding structures, mechanisms and habits. They may be classified broadly by their feeding habits as either detritivores, herbivores, omnivores, or carnivores (Moyle and Cech 2000; Horn et al. 2006). Within each category, organisms may be thought of as either euryphagous (eating a

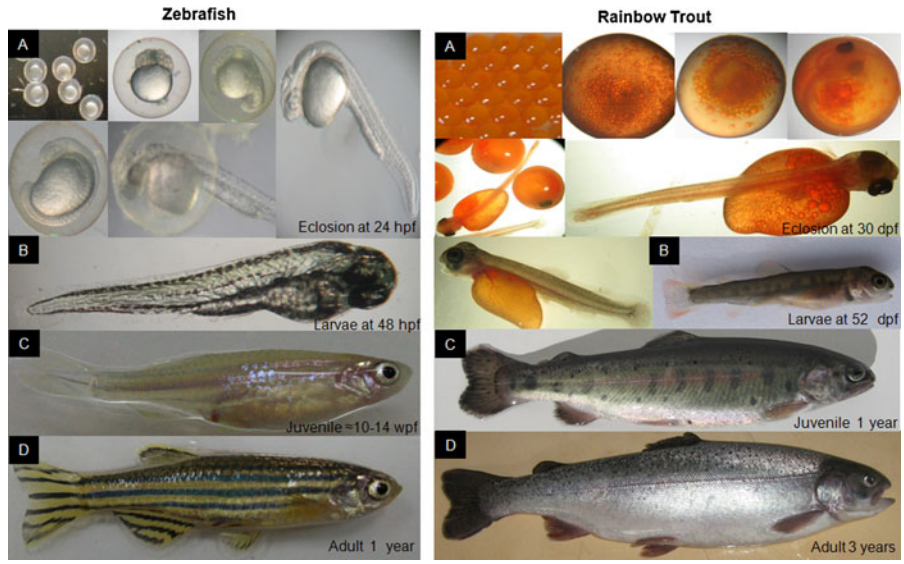

Fig. 2 Comparative view of the life-cycle of zebrafish and rainbow trout **a** Zebrafish eggs (1 mm of length), the different stages of embryo development at 28°C and eclosion at 24 hpf; Rainbow trout eggs (5 mm of length) the different stages of embryo development at 10°C, and eclosion at 30 dpf or 300 ATU. **b** Zebrafish larvae at 48 hpf (size ≈ 2 mm length); Rainbow trout larvae at 52 dpf or 520 ATU (size ≈ 2.5 cm length) **c** Juvenile zebrafish reach sexual maturity between 10–14 weeks post fertilization (size ≈ 21 mm length); Juvenile rainbow trout identifiable by their parr spots (size ≈ 20 cm length) **d** Adult zebrafish with common phenotype at ≈ 1 year old (size 3–5 cm length); Adult rainbow trout reach sexual maturity at 2–3 years old (size ≈ 40 cm length). The images of embryonic development (zebrafish and trout) were obtained with a Canon PowerShot SD550 digital camera and a stereoscopic zoom microscope Nikon SMZ-10 (20–160× in optical magnification)

great variety of foods), stenophagous (eating a limited variety of foods), or monophagous (eating only one type of food) (Moyle and Cech 2000). The majority of aquacultured fish are either euryphagous carnivores (such as salmon, bass, bream, halibut, turbot, flounders, and groupers), euryphagous omnivore (such as channel catfish, tilapia and zebrafish), or euryphagous herbivore (such as carp). The zebrafish has a long intestine with a large absorption area and no true stomach (Fig. 3), while carnivore fish species (salmon and trout) have a short intestine and a true stomach (Moyle and Cech 2000; revised in Rust 2002). Studies have shown that carnivorous fish, such as salmon, have a compensatory mechanism that allows them to modify and adapt their digestive and physiological systems in response, to a vegetal diet. Rainbow trout and sea bream, show gut augmentation when they are fed vegetal protein diets (over 75% fish meal replacement) (Santigosa et al. 2008). The vegetable diet decreases digestive activity, increaseds gut length and enzymatic activity of enzymes such as trypsin, and produceds a growth rate similar to fish fed with fish meal (Santigosa et al. 2008). Other

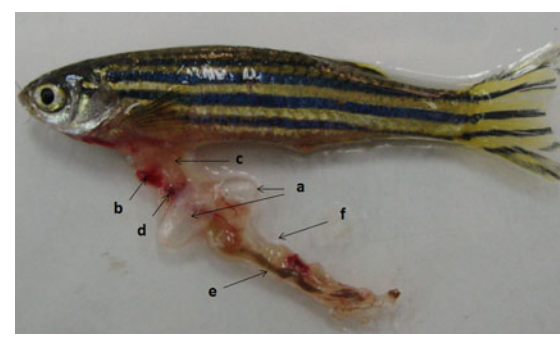

Fig. 3 Digestive tract from *Danio rerio*. (*a*) Swim bladder, (*b*) liver, (*c*) esophagus, (*d*) spleen, (*e*) intestine, and (*f*) pancreatic tissue (around the gut). The image was obtained with a Canon PowerShot SD550 digital camera under normal conditions employing the internal zoom (3× optical magnification)

evidence shows that gut length in fish is affected by ontogeny, diet and phylogeny. For example, gut length was measured in four fish species, two herbivores (*Cebidichtys violaceus* and *Xiphister mucosus*) and two carnivores (*X. atropurpureus* and *Anoplarchus purpurescens*), all fed with a

high-protein artificial diet (≥55% protein from marine animal sources). In herbivore species there was no ontogenetic change in gut dimensions on the high-protein artificial diet, suggesting that herbivores are genetically programmed to develop relatively large guts. However, *X. atropurpureus* (carnivore) increased its gut dimensions reaching a size similar to its sister taxon, *X. mucosus* (herbivore), suggesting a phylogenetic influence, but it also decreased its gut dimensions on the high-protein artificial diet, suggesting phenotypic plasticity (German and Horn 2006). These results show that the anatomical pattern of herbivore and carnivore vertebrates does not appear to be conserved in fishes, and show a phenotypic plasticity (ontogenetic increase) in the length of the digestive tract even in carnivorous species. These observations allow us to suppose that differences between the zebrafish digestive system and that of salmonids would not be a constraint when using the zebrafish as a model in nutritional studies.

Diet of the zebrafish

Information on the dietary preferences of wild zebrafish is limited. Apparently wild zebrafish are generalists, consuming a wide variety of benthic and planktonic crustaceans, worms and insect larvae (Dutta 1993). McClure et al. (2006) and Spence et al. (2007) analyzed gut contents of sampled zebrafish in natural habits and found that insects, mostly of terrestrial origin, were the predominant prey; the diet also included a small percentage of plankton**,** whose main components identified were zooplankton and insects (58.6%), invertebrate eggs and arachnids (2.5%), phytoplankton (5.9%) and other unidentified components (33%). Zebrafish in captivity may be fed with live prey such as brine shrimp (*Artemia* sp.), rotifers (*Brachionus* sp.), artificial diet, or some mixture of both (Watanabe et al. 1983). These results show that zebrafish can feed on animal and on vegetal protein sources, and suggests that they may have similar digestive mechanisms to both of herbivores such as carp and tilapia, and carnivores such as trout and salmon. It also suggests that they would be able to modify and adapt their digestive system in response to diet, which could facilitate the design of diets and feeding protocols that better reflect the specific digestive physiology and feeding behavior of the zebrafish.

Nutritional requirements of zebrafish

Fish nutritional requirements must be determined for each life stage: larva, juvenile, and adult, through controlled experiments that test the effects of different dietary components on survival, growth, disease/stress resistance and reproduction. Nutritional requirements are unknown for zebrafish, even though it is a widely used as a model organism in laboratories (Lawrence 2007). In the guide for laboratory use of zebrafish, specific nutritional data are not discussed (Westerfield 2000). This is due in part to the fact that zebrafish can be maintained and successfully spawned under a wide variety of diets and feeding regimes, including those that are likely sub-optimal (Lawrence 2007).

Lipids, particularly fatty acids (FA), are well known dietary requirements that supply energy and act as pheromones, hormones, and membrane components. Fish are unable to synthesize de novo polyunsaturated fatty acids (PUFA), including the essential ones such as linoleic (18:2 $\omega-6$) and linolenic acid (18:3 $\omega-3$). Therefore, fish must obtain these substances from their food. Cold water fish have a higher requirement for $\omega-3$ FA than $\omega-6$ FA, whereas warm water species require both $\omega-3$ and $\omega-6$ FA (Meinelt et al. 2000). Zebrafish, like many other warm-water fishes, require higher amounts of $\omega-6$ fatty acids (Watanabe 1981; Cowey and Sargent 1976). Meinelt et al. (1999, 2000) raised zebrafish on formulated feed containing varying ratios of $\omega-3$ and $\omega-6$ polyunsaturated fatty acids (PUFAs), and found that growth and fertilization rates were positively correlated with the level of $\omega-6$ PUFA in diet.

Protein requirements of juvenile omnivore, carnivore and herbivore ornamental fish are in accordance with the requirements reported for fish feeding (National Research Council 1993). The best approximation to assess zebrafish diet any requirements can probably be drawn from studies of cyprinid species such as the common carp and goldfish (*Carasius auratus*) (Lochman and Phillips 1996; Carvalho et al. 2004; Dabrowski et al. 2005). Protein requirements vary from 30 to 53% in diets for optimal growth in

goldfish (Fiogbé and Kestemont 1995). Westerfield (2000) proposed the feeding of adult zebrafish with a commercial diet of flake food for tropical fish; and for feeding larvae, the use of fine powder commercial food specific for these commercial foods have from 46 to 50% protein content. In relation to carbohydrates, Robison et al. (2008) examined the effect of manipulating dietary carbohydrate levels (0, 15, 25 and 35%) from the larval stage until sexual maturity in zebrafish. They demonstrated that long term manipulation of dietary carbohydrate levels had a significant effect on body weight gain, condition factor and body composition. More research on nutrient requirements of zebrafish is needed to gain comprehensive knowledge about to determine optimal dietary levels.

Growth rate in fishes

Growth is a quantitative trait and it may be measured at different times of the zebrafish life cycle. Many variations occur in zebrafish growth rates in laboratories (Eaton and Farley 1974b). This phenomenon is known as growth compensation or size-hierarchy effect (Brown 1957; Ricker 1958). It is particularly troublesome when using fish for experiments, where uniform growth and high survivability are important. Quantitative genetic variability of reproduction and growth performance using four zebrafish populations of different origins from Asia and Europe were studied. Traits of body length and the weight of progeny were measured every 2 weeks until 20 weeks of age. The average weight and length for the populations at 4, 12 and 20 weeks were 3.5; 74.9; 213.4 mg and 6.4; 15.3; 21.3 mm, respectively, with a high phenotypic correlation between the traits ($r = 0.95$) (Von Hertell et al. 1990). Goolish et al. (1999) assessed the effectiveness of 10 artificial diets. A good performance was achieved with a commercial diet, leading to a maximum larval survival of about 60% and a standard length of 6.4 mm at 21 dpf. Later Biga and Goetz (2006) reported an average total length of 4.7 mm at 28 dpf when only paramecia were used to feed larvae. Carvalho et al. (2006) demonstrated that zebrafish larvae could be reared without live food with an admissible growth and a high survival (7.9 mm and 84%, respectively), on the condition that food was presented in a continuous manner. When feeding larvae with artemia, the value obtained for standard length was 14.3 mm at 21 dpf, with a high larval survival of 86%. Santos et al. (2006) successfully used a mix of purified diet and artemia to feed zebrafish larvae, obtaining more than 70% survival at 120 dpf. These differences are indicative of discrepancies in rearing densities and in specifically diet, where growth is directly affected by nutrition and the feeding environment.

A quantitative trait such as growth is normally under control of polygenes, where each has a small effect on the phenotype, as well as a environmental influence and a possible influence of major genes and/or QTLs (Falconer and Mackay 1996). Little research has been done to identify growth QTLs in aquaculture fish (Araneda et al. 2008). However, in zebrafish QTLs for growth rate and muscle fat content have been identified on chromosome 23 (Wright et al. 2006). This result is valuable because it is the first step into identifing loci affecting these traits. Heritability estimations of growth rate in the main aquacultured fish species (salmon, trout, tilapia and carp) fluctuate from 20 to 41% (Tave 1993). Heritability estimated for weight and length at 4 and 20 weeks old in zebrafish were measured, were found to have very high values (0.17 and 0.40, respectively), (Von Hertell et al. 1990). This result agrees with estimates obtained in our laboratory for heritability of growth rate (wet weight) in 14 week-old zebrafish (0.42 ± 0.10) (Accini 2009).

Genes associated with growth in fishes

Growth traits have been under selection in fish and livestock breeding programs. Many genes associated with muscular growth (somatogenesis) have been mapped and sequenced, and have been shown to be conserved among mammals and fish (Lo Pestri et al. 2009). These results allowed the identification of numerous candidate genes involved in muscular growth of different fish species (De-Santis and Jerry 2007; Johnston et al. 2008). Among the many possible growth-regulating pathways in vertebrates, genes within the somatotropic axis and transforming growth factor superfamily have been the most targeted candidate genes in fish (De-Santis and Jerry 2007). The somatotropic axis essentially consists of growth hormone-releasing hormone (GHRH), growth

hormone (GH), growth hormone receptor (GHR), insulin-like growth factors (IGF-I and II), associated carrier proteins (IGFBPs) and receptors. These genes represent the main endocrine and autocrine regulators for skeletal muscle growth and are known to play a key role in the regulation of metabolism and of physiological processes (Moriyama et al. 2000; De-Santis and Jerry 2007; Johnston et al. 2008). The autocrine IGF-II transcription factor required for skeletal myocyte differentiation is regulated by *mTOR*, as well as by amino acid sufficiency. The mTOR-IGF axis is a molecular link between nutritional levels and skeletal muscle development (Erbay et al. 2003). In rainbow trout the evidence suggests that insulin and amino acid regulate TOR signaling (Seiliez et al. 2008). Another somatotropic axis hormone is leptin, which is thought to have a minor role in GH regulation. The presence of the *leptin* gene in teleosts initially was uncertain (Johnson et al. 2000), but now this peptide is known to be expressed in fishes and its DNA sequence has been found in the pufferfish and in the common carp (Kurokawa et al. 2005a, b; Huising et al. 2006). Structural growth in teleosts is mediated by growth factors (GFs) and myogenic regulatory factors (MRFs), generally produced in the muscle tissue (Tan et al. 2002).

Myostatin (MSTN), also known as growth/differentiation factor-8 (GDF-8), is a molecule belonging to the transforming growth factor β (TGF-β), which acts as a negative regulator of muscle development (McPherron et al. 1997). In fish, the MSTN mRNA has been detected in several tissues including gill, renal and gonadal tissues. These findings indicate that MSTN is involved in numerous physiological activities, and has been characterized in many fish species (rainbow trout, Atlantic salmon, sea bream, tilapia, catfish, among others) (De-Santis and Jerry 2007). *Mstn* is conserved as two genes in most teleosts (*Mstn 1 y Mstn 2*), as a result of the basal wide genome duplication (Maccatrozzo et al. 2001). In adult zebrafish subjected to environmental stress, both *Mstn-1* and *Mstn-2* genes expression were expressed in a wide variety of tissues (Helterline et al. 2006). Acosta et al. (2005) reported that the inactivation of the *Mstn* gene in zebrafish resulted in an increase of weight gain (45% more compared to the control), as was also observed in mice by McPherron et al. (1997). Previous studies in mammals have demonstrated that myostatin is binds to the two activin type II receptors (ActRII) (Lee and McPherron 2001; Rebbapragada et al. 2003). In zebrafish, ActRII and ActRIIB were shown to exhibit distinct roles in craniofacial development (Albertson et al. 2005). In Atlantic salmon, full-length ActRIIB cDNA was isolated and characterized by in situ hybridization and by RT–PCR in skeletal muscle (Ostbye et al. 2007). Carpio et al. (2009) examined the effect of administrating a recombinant soluble form of goldfish ActRIIB extracellular domain (G-ActRIIBed) to juvenile and larval goldfish, African catfish larvae (*Clarias gariepinus*) and tilapia larvae, it was found that this recombinant molecule stimulated growth in teleost fish in a dose-dependent manner.

Myogenic regulatory factors (MRFs) (myogenin, *Myod*, *myf-5*, *myf-4* or *myf-6*) constitute a small family of transcriptional factors, called the *Myod* gene complex, involved in myogenesis and are expressed exclusively in the skeletal muscle of vertebrates (Atchley et al. 1994). Genes belonging to the *Myod* complex have been characterized only in a few fish species such as in the common carp and in rainbow trout (Kobiyama et al. 1998; Johansen and Overturf 2005). In zebrafish, *myf-5* and *mrf-4* have been also identified (Chen et al. 2001; Hinits et al. 2007; Wang et al. 2008).

Another gene thought to be essential to growth in fish, is follistatin (*fst*). It is a secreted glycoprotein that was first identified as a potent inhibitor of follicle-stimulating hormone secretion which was explained by its strong binding affinity to the TGF-β protein activin (Phillips and Krestor 1998). Follistatin inhibits the transformation of growth factor-β proteins and is a known regulator of amniote myogenesis (Macqueen and Johnston 2008). Phylogenetic analyses suggest that one *fst* gene (*fst1*) is common to euteleosts, but a second gene (*fst2*) is conserved specifically within the Ostariophysi. Zebrafish *fst1* and *fst2* map respectively on chromosomes 5 and 10 in two genomic regions, and share a each conserved synteny to a region in tetrapods (Macqueen and Johnston 2008). Other growth genes studied in fish are those of the calpain-calpastatin (*CAST*) system. This system is important because it regulates a wide range of physiological processes including protein turnover and growth, cell cycle progression, and myoblast differentiation (Goll et al. 2003). Full-length cDNA has been obtained for *calpain 1* and *2* from rainbow trout, and show 65% identity to mouse

orthologues (Salem et al. 2005a). Also, *calpain 1*, *calpain 2* y *calpastatin* gene expression was measured in rainbow trout subjected to starvation for 35 days. Rainbow trout starvation resulted in the up-regulation of mRNA transcripts for these three genes (Salem et al. 2005a). Salem et al. (2005b) report in a second study, two *CAST* isoforms for rainbow trout: a long (*CAST-L*) and a short one (*CAST-S*). Lastly, genes from the ubiquitin-proteosome proteolytic pathway are considered to be major in route of protein degradation involved in skeletal muscle and are regulated by nutritional status. The identification of all of these genes contributes to the understanding of the mechanisms that regulate growth in non-mammalian vertebrates and suggests a powerful biotechnological approach to improve aquacultured fish growth; however, the expression of nutritional regulation of these growth trait genes is poorly documented in fishes.

Zebrafish nutritional genomics

Nutrigenomic studies are being directly developed in the most important aquacultured fish species using microarrays and RT–PCR analysis, which allows the observation of differential gene expression in response to diets containing proteins from different vegetal origins, and in response to diets of different oil compositions (Froystad et al. 2008, 2009; Lilleeng et al. 2007; Panserat et al. 2008; Douglas 2006). These nutrigenomic studies have generated basic knowledge about unchanged, over-expressed and under-expressed genes in response to different diets. Some nutrigenomic studies have observed the particular expression of some candidate genes correlated with growth in aquacultured fishes such as rainbow trout and Atlantic salmon under starvation and feeding conditions (Chauvigné et al. 2003; Salem et al. 2005a; Bower et al. 2008; Salem et al. 2007). Nevertheless, there has been little research on how fish diets affect the expression of genes that participate in growth. Chapalamadugu et al. (2009) evaluated the influence of dietary carbohydrate level (0, 15, 25, or 35%) on the temporal mRNA expression patterns (4, 8 or 12 week) of transcription factors that regulate satellite cell myocyte addition (MA) in rainbow trout. They showed that 15% and 25% carbohydrate containing diets significantly up- regulate *Myod* and *Myf5*, but not *Pax7* after 12 weeks of feeding. The high carbohydrate diet (35%) attenuated the increased mRNA expression of these transcription factors. These results suggest a potential role for satellite cells in nutrient sensing ability in fish. Recently Alami-Durante et al. (2010a) observed expression of related genes in white and red muscle of rainbow trout fed with graded levels of a mix of plant protein sources as substitutes for fishmeal. They showed changes in the expression of genes involved in lysosomal proteolysis in the white muscle. In another study Alami-Durante et al. (2010b) identified the amino acid profile and the changes in expression of myogenic regulatory factor genes and of myosin heavy chains in the muscle of juvenile rainbow trout when fed plant protein sources diets. They did not observe changes in the expression of genes *MyoD*, *MyoD2* or *myogenin* in lateral red muscle. However, they have demonstrated changes in the expression of the *MyoD* and of the fast-MHC genes in skeletal white muscle. These results indicate that the white and red muscles of rainbow trout are differently affected by nutritional changes.

Two studies in zebrafish have been performed with nutritional genomics. Robison et al. (2008) identified sexual dimorphism in hepatic gene expression in response to different percentages of carbohydrates in diets (0, 15, 25 and 35%) from the larval stage through sexual maturity. They observed substantial sexual dimorphism in the hepatic transcriptome-like genes associated with oxidative metabolism, carbohydrate metabolism and energy production. Drew et al. (2008) identified metabolic pathways regulated by starvation in the liver and in the brain of zebrafish in adult females after 21 days of starvation. They observed that starvation regulated gene expression of components of metabolic activity, reduced lipid metabolism, protein biosynthesis, caused proteolysis and cellular respiration. The response of the zebrafish hepatic transcriptome to starvation was strikingly similar to that of rainbow trout, and less similar to mouse, while the response of common carp differed considerably from the other three species. These results support the use of this fish as a model organism for nutritional genomic studies, where the results of these studies could be compared to similar question in aquacultured fish.

Conclusions and future perspectives

Zebrafish have several advantages that allow it to be used as a model organism for nutritional and growth studies, especially for nutritional genomic studies. Zebrafish possess the most developed genomic program compared to that of any other aquacultured fish, they are easy to maintain and breed, have short generational time and produce a large number of offspring. These advantages permit the use of the zebrafish as an excellent springboard for population studies and improving reproducibility of the experiments. Moreover, the zebrafish is evolutionally closer to the most important aquacultured fish than any other mammalian model organism, including the mouse. However, zebrafish can feed from animal and vegetal protein sources, which allows us to infer that it has nutritional pathways similar to those of cultivated herbivores such as carp and tilapia, and to those of carnivores such as trout and salmon. Nevertheless, more studies on zebrafish nutrition and protein, carbohydrates and lipid requirements are needed to assess the nutritional requirement for optimal skeletal muscle growth in fishes. New possible areas of investigation in zebrafish are needed in nutrition and nutrigenomics. Aquaculture investigation must take advantages of and find answers in model organisms such as the zebrafish, where studies of gene expression and genetic variants in response to nutrients could potentially be translational to aquacultured species. Studies in cultured species have been a major limitation because they require crosses to observe segregation of the different genetic variants, and the majority of these cultured fishes have a long generation time. In this case, the use of zebrafish as a model may be a good starting point to look for the genetic variants present in many genes. These genetic variants could be responsible for phenotypic variability of economically important traits such as growth. The identification of polymorphic sites in coding genes is a step forward to future investigations on any associations of these variants with the increase or reduction in the phenotypic expression of productive traits.

Acknowledgments This work was supported by the following fellowships to PU: Conicyt AT-24091052 and Doctoral fellowship from the Consorcio Empresarial de Genética y Desarrollo Biotecnológico para la Industria Salmonera (Aquainnovo S.A.) and, Grant DI I2 04/05-2 Universidad de Chile to CA. The authors acknowledge the help of Daniela Romo and Francisco Estay (Piscicola Hulilco Ltda.) for your assistance in photography. PU also wishes to thank the Programa de Doctorado en Ciencias de Recursos Naturales of the Universidad de La Frontera. We are especially grateful to Rashida Lathan for the English editing in the final version of this work.

References

Accini G (2009) Estimación de heredabilidad e identificación de marcadores RAPD asociados a tasa de crecimiento en zebrafish (*Danio rerio*). Tesis de Ingeniero Agrónomo, Facultad de Ciencias Agronómicas, Universidad de Chile, p 33

Acosta J, Carpio Y, Borroto I, Gonzalez O, Estrada M (2005) Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol 119:324–331

Alami-Durante H, Médale F, Cluzeaud M, Kaushik S (2010a) Skeletal muscle growth dynamics and expression of related genes in white and red muscle of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. Aquaculture 303:50–58

Alami-Durante H, Wrutniak-Cabello C, Kaushik SJ, Médale F (2010b) Skeletal muscle cellularuty and expression of myogenic regulatory factors and myosin heavy chains in rainbow trout (*Oncorhynchus mykiss*): effects of changes in dietary plant protein sources and amino acid profiles. Comp Biochem Physiol A 156:561–568

Albertson R, Payne-Ferreira T, Postlethwait J, Yelick P (2005) Zebrafish *acvr2a* and *acvr2b* exhibit distinct roles in craniofacial development. Dev Dyn 233:1405–1418

Aleström P, Holter J, Nourizadeh-Lillabadi R (2006) Zebrafish in functional genomics and aquatic biomedicine. Trends Biotechnol 24:15–21

Allendorf F, Thorgaard G (1984) Tetraploidy and the evolution of salmonid fishes. In: Turner B (ed) Evolutionary genetics of fishes. Plenum Press, New York, pp 1–46

Aparicio S, Chapman J, Stupka E, Putnam N, Chia J, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Sollewinjn Gelpke M, Roach J, Oh T, Ho IY, Wong M, Detter CH, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith S, Clark M, Edwards YJK, Doggett N, Zharkikh A, Tavtigian S, Pruss D, Barnstead M, Evans CH, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan H, Elgar G, Hawkins T, Venkatesh B, Rokhsar D, Brenner S (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science 297:1301–1310

Araneda C, Neira R, Lam N, Iturra P (2008) Salmonids. In: Kocher TD, Kole C (eds) Genome mapping and genomics in fishes and aquatic animals. Springer-Verlag, Berlin, Heidelberg, pp 1–43

Atchley W, Fitch W, Bronnerfraser M (1994) Molecular evolution of the Myod family of transcription factors. Proc Natl Acad Sci USA 91:11522–11526

Barbazuk W, Korf I, Kadavi I, Heyen J, Tate S, Wun E, Bedell J, McPherson J, Johnson S (2000) The syntenic

relationship of the zebrafish and human genomes. Genome Res 10:1351–1358
Bell M (2001) Lateral plate evolution in the threespine stickleback: getting nowhere fast. Genetica 1:445–461
Bergeron S, Milla L, Villegas R, Shen M-C, Burgess S, Allende M, Palma V, Karlstrom R (2008) Expression profiling identifies novel Hh/Gli regulated genes in developing zebrafish embryos. Genomics 91:165–177
Biga P, Goetz F (2006) Zebrafish and giant danio as models for muscle growth: determinate versus indeterminate growth as determined by morphometric analysis. Am J Physiol Regul Integr Comp Physiol 291:1327–1337
Boulding EG, Culling M, Glebe B, Berg PR, Lien S, Moen T (2008) Conservation genomics of Atlantic salmon: SNPs associated with QTLs for adaptative traits in parr from four trans-Atlantic backcrosses. Heredity 101:381–391
Bower N, Li X, Taylor R, Johnston A (2008) Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. J Exp Biol 211:3859–3870
Bradley K, Elmore B, Breyer J, Yaspan B, Jessen J, Knapik E, Smith J (2007) A major zebrafish polymorphism resource for genetic mapping. Genome Biol 8:55–65
Breder C, Rosen D (1966) Modes of reproduction in fishes. The Natural History Press, New York, p 941
Briggs J (2002) The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol 282:R3–R9
Brown M (1957) Experimental studies on growth. In: Brown M (ed) The physiology of fishes. Academic Press, New York, pp 261–400
Carpio Y, Acosta J, Morales R, Santisteban Y, Sanchéz A, Estrada M (2009) Regulation of body mass growth through activin type IIB receptor in teleosts fish. Gen Comp Endocrinol 160:158–167
Carvalho A, Sá R, Oliva-Teles A, Bergot P (2004) Solubility and peptide profile affect the utilization of dietary protein by common carp (*Cuprinus carpio*) during early larval stages. Aquaculture 234:319–333
Carvalho A, Araujo L, Santos M (2006) Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical, and a purified starter diet on larval performance. Aquacult Res 37:1107–1111
Cenadelli S, Maran V, Bongioni G, Fusetti L, Parma P, Aleandri R (2007) Identification of nuclear SNPs in gilthead seabream. J Fish Biol 70:399–405
Chapalamadugu K, Robison B, Drew R, Powell M, Hill R, Amberg J, Rodnick K, Hardy R, Hill M, Murdoch G (2009) Dietary carbohydrate level affects transcription factor expression that regulates skeletal muscle myogenesis in rainbow trout. Comp Biochem Physiol B 153:66–72
Chauvigné F, Gabillard J, Weil C, Rescan P (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6 and myostatin mRNA expression in rainbow trout myotomal muscle. Gen Comp Endocrinol 132:209–215
Chen Y, Lee W, Liu C, Tsai H (2001) Molecular structure, dynamic expression, and promoter analysis of zebrafish (*Danio rerio*) Myf-5 gene. Genesis 29:22–35
Chen F, Lee Y, Jiang Y, Wang S, Peatman E, Abernathy J, Liu H, Liu SK, Kucuktas H, Ke CH, Liu Z (2010) Identification and characterization of Full-Length cDNAs in Channel Catfish (*Ictalurus punctatus*) and Blue Catfish (*Ictalurus furcatus*). Plos One 12e: 11543
Clark M (2003) Genomics and mapping of teleostei. Comp Funct Genomics 4:182–193
Cossins A, Crawford D (2005) Fish as model for environmental genomics. Nat Rev Genet 6:324–340
Cowey C, Sargent J (1976) Lipid nutrition in fish. Comp Biochem Physiol 57B:269–273
Cresko W, McGuigan K, Phillips P, Postlethwait J (2007) Studies of threespine stickleback developmental evolution: progress and promise. Genetica 129:105–126
Crollius H, Weissenbach J (2008) Fish genomics and biology. Genome Res 15:1675–1682
Dabrowski K, Hliwa P, Gomulka P, Sienicki M, Carvalho AP, Ostaszewska T, Terjesen B (2005) Studies on the utilization of free amino acid, casein-based, or commercial diets in rearing common carp larvae. Abstr of Aquac Am 344, New Orleans, USA
Daga R, Thode G, Amores A (1996) Chromosome complement, C-banding, Ag-NOR and replication banding in the zebrafish *Danio rerio*. Chromosome Res 4:29–32
Dahm R (2002) Atlas of embryonic stages of development in the zebrafish. In: Nusslein-Volhard C, Dahm R (eds) Zebrafish: a practical approach. Oxford University Press, Oxford, pp 219–236
Dahm R, Geisler R (2006) Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol 0:1–17
Davidson W, Koop B, Jones S, Iturra P, Vidal R, Maass A, Jonassen I, Lien S, Omholt S (2010) Sequencing the genome of the Atlantic salmon (*Salmo salar*). Genome Biol 11:403
Davis GP, Hetzel DJ (2000) Integrating molecular genetics technology with traditional approaches for genetic improvement in aquaculture species. Aquacult Res 31:3–10
De-Santis C, Jerry D (2007) Candidate growth genes in finfish—Where should we be looking? Aquaculture 272:22–38
Dominik S, Henshall J, Kube P, King H, Lien S, Kent M, Elliott N (2010) Evaluation of an Atlantic salmon SNP chip as a genomic tool for the application in a Tasmanian Atlantic salmon (*Salmo salar*) breeding population. Aquaculture 308:S56–S61
Douglas S (2006) Microarray studies of genes expression in fish. J Integr Biol 10(4):474–489
Drew R, Rodnick K, Settles M, Wacyk J, Churchill E, Powell M, Hardy R, Murdoch G, Hill R, Barrie R (2008) Effect of starvation on the transcriptomes of the brain and liver in adult female zebrafish. Physiol Genomics 35:283–295
Driever W, Stemple D, Schier A, Solnica-Krezel L (1994) Zebrafish: genetic tools for studying vertebrate development. Trends Genet 10:152–159
Du SH (2004) Molecular regulation of fish muscle development and growth. In: Gong Z, Korzh V (eds) Molecular aspects of fish and marine biology, volume 2, fish development and genetics the zebrafish and medaka models. World Scientific, Singapure, pp 581–611
Dumas A, Frane J, Bureau D (2010) Modelling growth and body composition in fish nutrition: where have we been and where are we going? Aquacult Res 41:161–181

Dutta S (1993) Food and feeding habits of *Danio rerio* (Ham. Buch.) inhabiting gadigarh stream, Jammu. J Freshw Biol 5:165–168

Eaton R, Farley R (1974a) Spawning cycle and egg production of zebrafish, *Brachydanio rerio*, in the laboratory. Copeia 1:195–204

Eaton R, Farley R (1974b) Growth and the reduction of depensation of zebrafish, *Brachedanio rerio* reared in the laboratory. Copeia 1:204–209

Ehrlich J, Sankoff D, Nadeau J (1997) Synteny conservation and chromosome rearrangements during mammalian evolution. Genetics 147:289–296

Erbay E, Park I, Nuzzi P, Schoenherr C, Chen J (2003) IGF-II transcription in skeletal myogenesis is controlled by *mTOR* and nutrients. J Cell Biol 163:931–936

Estay F, Cerisola H, Téllez V (1994) Biologia del desarrollo y reproducción artificial en la trucha arcoíris. Publicación del grupo de investigación de la Universidad de Chile, Universidad Católica de Valparaíso y del Instituto Fomento Pesquero, Chile, pp 1–28

Falconer D, MacKay T (1996) Introduction to quantitative genetics. Longman Group Ltda, Malasia, p 464

Fiogbé E, Kestemont P (1995) An assessment of the protein and amino acid requirement in goldfish (*Carassius auratus*) larvae. J Appl Ichthyol 11:282–289

Fjalestad KT, Moen T, Gomez-Raya L (2003) Prospects for genetic technology in salmon breeding programmes. Aquacult Res 34:397–406

Franch R, Louro B, Tsalavouta M, Chatziplis D, Tsigenopoulos S, Sorropoulou E, Antonello J, Magoulas A, Mylonas C, Babbucci M, Patarnello T, Power D, Kotoulas G, Bargelloni L (2006) A genetic linkage map of the hermaphrodite teleost fish *Sparus aurata* L. Genetics 174:851–861

Froystad M, Lilleeng E, Bakke-Mckellep A, Vekterud K, Hemre G, Krogdahl A (2008) Gene expression in distal intestine of Atlantic salmon (*Salmo salar* L.) fed genetically modified soybean meal. Aquacult Nutr 14:204–214

Froystad M, Lilleeng E, Bakke-Mckellep A, Vekterud K, Valen E, Hemre G, Krogdahl A (2009) Distal intestinal gene expression in Atlantic salmon (*Salmo salar* L.) fed genetically modified maize. Aquacult Nutr 15:104–115

German D, Horn M (2006) Gut length and mass in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Mar Biol 148:1123–1134

Goll D, Thompson V, Li H, Wei W, Cong J (2003) The calpain system. Physiol Rev 83:731–801

Goolish E, Okutake K, Lesure S (1999) Growth and Survivorship of larval zebrafish *Danio rerio* on processed diets. N Am J Aquacult 61:189–198

Gornung E, Gabrielli I, Cataudella S, Sola L (1997) CMA_3—banding pattern and fluorescence in situ hybridization with 18S rRNA genes in zebrafish chromosomes. Chromosome Res 5:40–46

Govoroun M, Le Gac F, Guiguen Y (2006) Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalized rainbow trout cDNA libraries. BCM Genomics 7:196

Grunwald D, Eisen J (2002) Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev Genet 3:717–724

Hahlbeck E, Katsiadaki I, Mayer I, Adolfsson-Erici M, James J, Bengtsson B (2004) The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II-kidney hypertrophy, vitellogenin and spiggin induction. Aquat Toxicol 70:311–326

Hardy R (2010) Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult Res 41:770–776

Hayes B, Laerdahl JK, Lien S, Moen T, Berg P, Hindar K, Davison WS, Koop BF, Adzhubei A, Hoyheim B (2007) An extensive resources of single nucleotide polymorphism markers associated with Atlantic salmon (*Salmo salar*) expressed sequences. Aquaculture 265:82–90

Hedges S (2002) The origin and evolution of model organisms. Nat Rev Genet 3:838–843

Helterline D, Garikipati D, Stenkamp D, Rodgers D (2006) Embryonic and tissue-specific regulation of myostatin-1 and-2 gene expression in zebrafish. Gen Comp Endocrinol 151(1):90–97

Hinegardner R, Rosen DE (1972) Cellular DNA content and the evolution of teleostean fishes. Am Nat 106:621–644

Hinits Y, Osborn D, Carvajal J, Rigby P, Hughes M (2007) *Mrf4* (*myf6*) is dynamically expressed in differentiated zebrafish skeletal muscle. Gene Expr Patterns 7:738–745

Horn M, Gawlicka A, German D, Logothetis E, Cavanagh J, Boyle K (2006) Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory. Mar Biol 149:1237–1245

Huising M, Geven E, Kruiswijk C, Nabuurs S, Stolte E, Spanings F, Verburg-van Kemenade B, Flik G (2006) Increased leptin expression in common carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. Endocrinology 147:5786–5797

Janvier P (1996) Early vertebrates. Oxford Science Publications, Clarendon Press, New York 375

Johansen K, Overturf K (2005) Sequence, conservation, and quantitative expression of rainbow trout Myf-5. Comp Biochem Physiol B: Biochem Mol Biol 140:533–541

Johansen S, Coucheron D, Andreassen M, Ove Karlsen B, Furmanek T, Jorgensen T, Emblem A, Breines R, Nordeide J, Moun T, Nederbragt A, Stenseth N, Jakobsen K (2009) Large-scale sequence analyses of Atlantic cod. New Biotechnol 25:263–271

Johnson R, Johnson T, Londraville R (2000) Evidence for leptin expression in fishes. J Exp Zool A: Comp Exp Biol 286:718–724

Johnston I (1999) Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177:99–115

Johnston I, Macqueen D, Watabe S (2008) Molecular biotechnology of development and growth in fish muscle. In: Tsukamoto K, Kawamura T, Takeuchi T, Douglas Beard T, Kaiser M (eds) Fisheries for global welfare and environment. 5th World Fisheries Congress, Scotland, pp 241–262

Kaput J, Rodriguez RL (2003) Nutritional Genomics: the next frontier in the postgenomic era. Physiol Genomics 16:166–177

Kasahara M, Naruse K, Sasaki SH, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shamida A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa SH, Shimizu N, Hashimoto SH, Jun Yang, Lee Y, Matsushima K, Sugano S, Sakaisumi M, Takanori N, Ohishi K, Haga SH, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita SH, Kohara Y (2007) The medaka draft genome and insigts into vertebrate genome evolution. Nature 447:714–719

Katsiadaki I, Scott AP, Mayer I (2002) The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. Mar Environ Res 54:725–728

Kimmel C, Ballard W, Kimmel S, Ullmann B, Schilling T (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310

Kobiyama A, Nihei Y, Hirayama Y, Kikuchi K, Suetake H, Johnston I, Watabe S (1998) Molecular cloning and developmental expression patterns of the MyoD and MEF2 families of muscle transcription factors in the carp. J Exp Biol 201:2801–2813

Kurokawa T, Uji S, Suzuki T (2005a) Identification of pepsinogen gene in the genome of stomachless fish. Takifugu rubripes Comp Biochem and Physiol Part B: Biochem Mol Biol 140:133–140

Kurokawa T, Uji S, Suzuki T (2005b) Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides 26:745–750

Lauder G, Liem K (1989) The evolution and interrelationships of the Actinopterygian fishes. Bull Mus Comp Zool 150:95–187

Lawrence C (2007) The husbandry of zebrafish (*Danio rerio*): a review. Aquaculture 269:1–20

Lee S, McPherron A (2001) Regulation of myostatin activity and muscle growth. PNAS 16:9306–9311

Leong J, Jantzen S, Von Schalburg K, Cooper G, Messmer A, Liao N, Munro S, Moore R, Holt R, Jones S, Davison W, Koop B (2010) *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. BCM Genomics 11:279

Li P, Peatman E, Wang SH, Feng J, He CH, Baoprsertkul P, Xu P, Kucuktas H, Nandi S, Somridhivej B, Serapion J, Simmons M, Turan C, Liu L, Muir W, Dunham R, Brady Y, Grizzle J, Liu Z (2007) Towards the ictalurid catfish transcriptome: generation and analysis of 31, 215 catfish ESTs. BCM Genomics 8:117

Lilleeng E, Froystad M, Ostby G, Valen E, Krogdahl A (2007) Effects of diet containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (*Salmo salar* L.). Comp Biochem Physiol Part A: Mol Integr Physiol 147:25–36

Liu Z, Cordes J (2004) DNA marker technologies and their applicationsin aquaculture genetics. Aquaculture 238:1–37

Liu H, Jiang Y, Wang SH, Ninwichian P, Somridhivej B, Xu P, Abernathy J, Kucuktas H, Liu Z (2009) Comparative analysis of catfish BAC end sequences with the zebrafish genome. BCM Genomics 10:592

Lo Pestri R, Lisa C, Di Stasio L (2009) Molecular genetics in aquaculture. Ital J of Anim Sci 8:299–313

Lochman R, Phillips H (1996) Nutrition and feeding of baitfish. Aquacult Mag 4:87–89

Lu J, Peatman E, Yang Q, Wang SH, Hu Z, Reecy J, Kucuktas H, Liu Z (2010) The catfish genome database cBARBEL: an informatics platform for genome biology of ictalurid catfish. Nucleic Acids Res 39:D815–D821

Maccatrozzo L, Bargelloni L, Cardazzo B, Rizzo G, Patarnello T (2001) A novel second myostatin gene is present in teleost fish. FEBS Lett 509:36–40

Macqueen D, Johnston I (2008) Evolution of follistatin in teleosts revealed through phylogenetic, genomic and expression analyses. Dev Genes Evol 218:1–14

Marti M, Moreno-Aliaga M, Zulet A, Martinez J (2005) Avances en nutrición molecular: nutrigenómica y nutrigenética. Nutr Hosp 3:157–164

McClure M, McIntyre P, McCune A (2006) Notes on the natural diet and habitat of eight danioin fishes, including the zebrafish *Danio rerio*. J Fish Biol 69:553–570

McPherron A, Lawler A, Lee S (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83–90

Meinelt T, Schultz C, Worth M, Kurzinger H, Steinberg C (1999) Dietary fatty acid composition influences the fertilization rate of zebrafish (*Danio rerio* Hamilton-Buchanon). J Appl Ichthyol 15:19–23

Meinelt T, Schultz C, Worth M, Kurzinger H, Steinberg C (2000) Correlation of diets high in n6-polyunsaturated fatty acids with high growth rate in zebrafish (*Danio rerio*). Comp Med 50:43–45

Meli R, Prasad A, Patowary A, Lalwani K, Maini J, Sharma M, Singh A, Kumar G, Jadhav V, Scaria V, Sivasubbu S (2008) FishMap: a community resource for zebrafish genomics. Zebrafish 5:125–130

Metscher B, Ahlberg P (1999) Zebrafish in context: uses of a laboraty model in comparative studies. Dev Biol 210:1–4

Moen T, Hayes B, Baranski M, Berg P, Kjoglum S, Koop B, Davidson W, Omholt S, Lien S (2008a) A linkage map of the Atlantic salmon (*Salmo salar*) based on EST-derived SNP markers. BCM Genomics 9:223–237

Moen T, Hayes B, Nilsen F, Delghandi M, Fjalestad K, Fevolden SE, Berg PR, Lien S (2008b) Identification and characterization of novel SNP markers in Atlantic cod: evidence for directional selection. BMC Genetics 9:18

Mommsen T (2001) Paradigms of growth in fish. Comp Biochem Physiol B 129:207–219

Moriyama S, Ayson F, Kawauchi H (2000) Growth regulation by insulin-like growth factor-I in fish. Biosci Biotechnol Biochem 64(8):1553–1562

Moyle P, Cech J (2000) Fishes. An introduction to ichthyology. Prentice Hall Inc., USA, p 612

Müller M, Kersten S (2003) Nutrigenomics: goals and strategies. Nat Rev Genet 4:315–322

Mutch M, Wahli W, Williamson G (2005) Nutrigenomics and nutrigenetics: the emerging faces of nutrition. The FASEB J 19:1602–1615

Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) A medaka gen map: the trace of ancestral

vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res 14:820–828

Nielsen E, Hansen M, Meldrup D (2006) Evidence of microsatellite hitch-hiking selection in Atlantic cod (*Gadus morhua* L.): implications for inferring population structure in nonmodel organisms. Mol Ecol 15:3219–3229

NRC National Research Council (1993) Nutrient requeriments of coldwater fishes. National Academy of Science, Washington, DC USA 63

Ojima Y (1983) Fish cytogenetics. Suiko-sha, Tokyo, Japan 453

Orban L, Wu Q (2008) Cyprinids. In: Kocher TD, Kole C (eds) Genome mapping and genomics in fishes and aquatic animal, volume 2. Springer-Verlag, Berlin, Heidelberg, pp 45–97

Ostbye T, Bardal T, Vegusdal A, Frang O, Kjorsvik E, Andersen O (2007) Molecular cloning of the Atlantic salmon activin receptor IIB cDNA-Localization of the receptor and myostatin in vivo and in vitro in muscle cells. Comp Biochem Physiol 2:101–111

Panserat S, Kaushik S (2010) Regulation of gene expression by nutritional factors in fish. Aquacult Res 41:751–762

Panserat S, Kirchner S, Kaushik S (2007) Nutrigenomics. In: Nakagawa H, Sato M, Gatlin D III (eds) Dietary supplements for the health and quality of cultured fish. CAB International North America, USA, pp 210–229

Panserat S, Ducasse-Cabanot S, Plagnes-Juan E, Srivastava P, Kolditz C, Piumi F, Esquerré D, Kaushik S (2008) Dietary fat level modifies the expression of hepatic genes in juvenile rainbow trout (*Oncorhynchus mykiss*) as revealed by microarray analysis. Aquaculture 275:235–241

Phillips D, Krestor D (1998) Follistatin: a multifunctional regulatory protein. Front Neuroendocrinol 19:287–322

Postlethwait J (2004) Evolution of the zebrafish genome. In: Gong Z, Korzh V (eds) Molecular Aspects of Fish and Marine Biology, volume 2, fish development and genetics the zebrafish and medaka models. World Scientific, Singapure, pp 581–611

Quinn N, Levenkova N, Chow W, Bouffard P, Boroevich K, Knight J, Jarvie T, Lubieniecki K, Desany B, Koop B, Harkins T, Davidson W (2008) Assessing the feasibility of GS FLX Pyrosequencing for sequencing the Atlantic salmon genome. BMC Genomics 9:404

Rebbapragada A, Benchabane H, Wrana J, Celeste A, Attisano L (2003) Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230–7242

Reid DP, Santo A, Glebe B, Danzmann RG, Ferguson M (2005) QTL for body weight and condition factor in Atlantc salmon (*Salmo salar*): comparative analysis with rainbow trout (*Oncorhynchus mykiss*) and Artic charr (*Salvelinus alpinus*). Heredity 94:166–172

Rexroad CE III, Lee Y, Keele JW, Karamycheva S, Brown G, Koop B, Gahr SA, Palti Y, Quackenbush J (2003) Sequence analysis of a rainbow trout cDNA library and creation of a gene index. Cytogenet Genome Res 102: 347–354

Rexroad C III, Rodriguez M, Coulibaly I, Gharbi K, Danzmann R, DeKoning J, Phillips R, Palti Y (2005) Comparative mapping of expressed sequence tags containing microsatellites in rainbow trout (*Oncorhynchus mykiss*). BCM Genomics 6:54–62

Rexroad C III, Palti Y, Gahr S, Vallejo R (2008) A second generation genetic map for rainbow trout (*Oncorhynchus mykiss*). BCM Genet 9:74–88

Ricker, W (1958) Hanbook of computations for biological statistics of fish populations. Bull Fish Res Bd Canada, p 300

Robison B, Drew R, Murdoch G, Powell M, Rodnick K, Settles M, Stone D, Churchill E, Hill R, Papasani M, Lewis S, Hardy R (2008) Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*). Comp Biochem Physiol Part D 2:141–154

Roush W (1996) Zebrafish embryology builds a better model vertebrate. Science 272:1103

Rust M (2002) Nutritional physiology. In: Halver J, Hardy R (eds) Fish nutrition, 3rd edn. Academic press, USA, pp 368–446

Ryynänen HJ, Primmer C (2006) Single nucleotide polymorphism (SNP) discovery in duplicated genomes: intron-primed exon crossing (IPEC) as a strategy for avoiding amplification of duplicated loci in Atlantc salmon (*salmo salar*) and other salmon fishes. BCM Genomics 7:192

Salem M, Nath J, Rexroad C, Killefer J, Yao J (2005a) Identification and molecular characterization of the rainbow trout calpains (*Capn1* and *Calp2*): their expression in muscle wasting during starvation. Comp Biochem Physiol B: Biochem Mol Biol 140:63–71

Salem M, Yao J, Rexroad C, Kenney P, Semmens K, Killefer J, Nath J (2005b) Characterization of calpastatin gene in fish: its potential role in muscle growth and fillet quality. Comp Biochem Physiol B: Biochem Mol Biol 141:488–497

Salem M, Silverstein J, Rexroad C III, Yao J (2007) Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). BCM Genomics 8:328–344

Santigosa E, Sánchez J, Médale F, Kaushik S, Peréz-Sánchez J, Gallardo M (2008) Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture 282:68–74

Santos M, Micael J, Carvalho A, Morabito R, Booy P, Massanisso P, Lamoree M, Reis-Henriques M (2006) Estrogens counteract the masculinizing efect of tributyltin in zebrafish. Comp Biochem and Physiol C: Toxicol Pharmacol 142:151–155

Sarropoulou E, Power DM, Magoulas A, Geisler R, Kotoulas G (2005) Comparative analysis and characterization of expressed sequence tags in gilthead sea bream (*Sparus aurata*) liver and embryos. Aquaculture 243:69–81

Sarropoulou E, Franch R, Louro B, Power D, Bargelloni L, Magoulas AN, Senger F, Tsalavouta M, Patarnello T, Galibert F, Kotoulas G, Geisler R (2007) A gene-based radiation hybrid map of the gilthead sea bream *Sparus aurata* refines and exploits conserved synteny with *Tetraodon nigroviridis*. BCM Genomics 8:44

Seiliez I, Gabillard J, Skiba-Cassy S et al (2008) An in vivo and in vitro assessment of TOR signaling cascade in

rainbow trout (Onchorinchus mykiss). Am J Physiol Regul Integr Comp Physiol 295:329–335

Senger F, Priat C, Hitte CH, Sarropoulou E, Franch R, Geisler R, Bargelloni L, Power D, Galibert F (2006) The first radiation hybrid map of perch-like fish: the gilthead seabream (*Sparus aurata* L.). Genomics 87:793–800

Smith C, Elfstrom C, Seeb L, Seeb J (2005) Use of sequence data from rainbow trout and Atlantic salmon for SNP detection in Pacific salmon. Mol Ecol 14:4193–4203

Spence R, Fatema M, Ellis S, Ahmed Z, Smith C (2007) Diet, growth and recruitment of wild zebrafish in Bangladesh. J Fish Biol 71:304–309

Stickney H, Schmutz J, Woods I, Holtzer C, Dickson M, Kelly P, Myers R, Talbot W (2002) Rapid mapping of zebrafish mutations with SNPs and oligonuleotide microarrays. Genome Res 12:1929–1934

Tan X, Hoang L, Du S (2002) Characterization of muscle-regulatory genes, Myf5 and myogenin, from striped bass and promoter analysis of muscle-specific expression. Mar Biotechnol 4:537–545

Tave D (1993) Genetics for fish hatchery managers. An AVI book, New York, p 415

Thorsen J, Zhu B, Frengen E, Osoegawa K, Jong PJ, Koop BF, Davidson W, Hoyheim B (2005) A highly redundant BAC library of Atlantic salmon (*Salmo salar*): an important tool for salmon projects. BCM Genomics 6:50

Turchini G, Torstensen B, Ng W-K (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1:10–57

Vignal A, Milan D, San Cristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. Genet Sel Evol 34:275–305

Volff J (2005) Genome evolution and biodiversity in teleost fish. Heredity 94:280–294

Von Hertell U, Hörstgen-Schwarrk G, Langholz H, Jung B (1990) Family studies on genetic variability in growth and reproductive performance between and within test fish populations of the zebrafish, *Brachydanio rerio*. Aquaculture 85:307–315

Wang Y, Li C, Lee G, Tsay H, Tsai H, Chen Y (2008) Inactivation of zebrafish mrf4 leads to myofibril misalignment and motor axon growth disorganization. Dev Dyn 237:1043–1050

Wang S, Peatman E, Abernathy J, Waldbieser G, Lindquist E, Richardson P, Lucas S, Wang M, Li P, Thimmapuram J, Liu L, Vullaganti D, Kucuktas H, Murdock CH, Small B, Wilson M, Liu H, Jiang Y, Lee Y, Chen F, Lu J, Wang W, Xu P, Somridhivej B, Baoprasertkul P, Quilang J, Sha Z, Bao B, Wang Y, Wang Q, Takano T, Nandi S, Liu S, Wong L, Kaltenboeck L, Quiniou S, Bengten E, Miller N, Trant J, Rockhsar D, Liu Z, The Catfish Genome Consortium (2010) Assembly of 500, 000 inter-specific catfish expressed sequence tags and large scale gene-associated marker development for whole genome association studies. Genome Biol 11:R8

Watanabe T (1981) Lipid nutrition in fish. Comp Biochem Physiol B 73:3–15

Watanabe T, Kitajima C, Fujita S (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. Aquaculture 34:115–145

Wegrzyn D, Ortubay S (2009) Salmonidos en patagonia, volumen 1. Mar del plata, Argentina, p 164

Wesmajervi MS, Tafese T, Stenvik J, Fjalested KT, Damsgard B, Delghandi M (2007) Eight new microsatellite markers in Atlantic cod (*Gadus morhua* L.) derived from an enriched genomic library. Mol Ecol 7:138–140

Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon Press, Eugene, Oregon USA, p 385

Wittbrodt J, Shima A, Schartl M (2002) Medaka—a model organism from the far east. Nat Rev Genet 3:53–64

Woods G, Wilson C, Friedlander B, Chang P, Reyes K, Nix R, Kelly D, Chu F, Postlethwait H, Talbot W (2005) The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res 15:1307–1314

Wright D, Nakamichi R, Krause J, Butlin R (2006) QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). Behav Genet 36:271–284

Xu P, Wang SH, Liu L, Thorsen J, Kukuktas H, Liu Z (2007) A BAC-based physical map of the channel catfish genome. Genomics 90:380–388