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ORIGINAL ARTICLE

RNA Sequencing to Study Gene Expression and SNP Variations Associated with Growth in Zebrafish Fed a Plant Protein-Based Diet

Pilar E. Ulloa · Gonzalo Rincón · Alma Islas-Trejo · Cristian Araneda · Patricia Iturra · Roberto Neira · Juan F. Medrano

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Abstract The objectives of this study were to measure gene expression in zebrafish and then identify SNP to be used as potential markers in a growth association study. We developed an approach where muscle samples collected from low- and high-growth fish were analyzed using RNA-Sequencing (RNA-seq), and SNP were chosen from the genes that were differentially expressed between the low and high groups. A population of 24 families was fed a plant protein-based diet from the larval to adult stages. From a total of 440 males, 5 % of the fish from both tails of the weight gain distribution were selected. Total RNA was extracted from individual muscle of 8 low-growth and 8 high-growth fish. Two pooled RNA-Seq libraries were prepared for each phenotype using 4 fish per library. Libraries were sequenced using the Illumina GAII Sequencer and analyzed using the CLCBio genomic workbench software. One hundred and twenty-four genes were differentially expressed between phenotypes (p value$<$0.05 and FDR$<$0.2). From these genes, 164 SNP were selected and genotyped in 240 fish samples. Marker-trait analysis revealed 5 SNP associated with growth in key genes (*Nars*, *Lmod2b*, *Cuzd1*, *Acta1b*, and *Plac8l1*). These genes are good candidates for further growth studies in fish and to consider for identification of potential SNPs associated with different growth rates in response to a plant protein-based diet.

Keywords Zebrafish · Gene expression · Single nucleotide polymorphism · Dietary plant proteins · Growth association analysis

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P. E. Ulloa
Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

P. E. Ulloa (✉) · C. Araneda · R. Neira
Departamento de Producción Animal, Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile
e-mail: pilarelizabeth@gmail.com

P. Iturra
Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70061-7, Santiago, Chile

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

G. Rincón · A. Islas-Trejo · J. F. Medrano
Department of Animal Science, University of California-Davis, One Shields Ave, Davis, CA 95616, USA

Present Address:
P. E. Ulloa
Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Andrés Bello, República 217, Santiago 8370146, Chile

Present Address:
G. Rincón
Zoetis, VMRD Genetics R&D, 333 Portage Street, Kalamazoo, MI 49007, USA

Introduction

Aquaculture is the fastest growing food production sector worldwide. Between 1970 and 2010, international farmed fish production grew at an average rate of 8.8 % per year, reaching a high of 60 million tons (excluding aquatic plants and non-food products) with an estimated total value of US$119,000 million (FAO 2012). However, wild fish capture to produce fish meal and fish oil has overexploited the oceans, with a consequent reduction in yield from 10.7 million tons in 2004 to just 4.2 million tons in 2010 (FAO 2012). As a result, sharp

rises in the price of fish meal and fish oil to supply the high demand for manufactured aquafeed have reduced the profitability of aquaculture, and over fishing negatively impacts the environment and natural resources (Bostock et al. 2010). Therefore, the aquaculture industry has developed diets that include plant protein (from 25 to 50 % replacement), reducing feed costs and environmental impact (Hardy 2010). The most common plant protein incorporated into fish diet has been soy flour. However, the use of corn, peas, lupins, canola, barley, and wheat has gradually increased (Naylor et al. 2009). To maintain current levels of fish production, it is anticipated that in the future, aquaculture activity will depend heavily on plant protein in the diet (FAO 2012). Key challenges will be to transform carnivorous fish (such as salmonids) into more herbivorous fish and to cultivate strains that tolerate higher levels of plant protein in their diet.

Many studies have revealed that incorporating large amounts of soy meal into the diet (50, 75, and 100 %) decreases growth in farmed fish, including common carp (*Cyprinus carpio*) (Pongmaneerat et al. 1993), Nile tilapia (*Oreochromis niloticus*) (Fontaínhas-Fernandes et al. 1999), seabream (*Sparus aurata*) (Gómez-Requeni et al. 2004), Atlantic salmon (*Salmo salar*) (Mundheim et al. 2004), and rainbow trout (*Oncorhynchus mykiss*) (Médale et al. 1998; Vilhelmsson et al. 2004; Alami-Durante et al. 2010). Our group recently demonstrated the same response in zebrafish (Ulloa et al. 2013). This response has been primarily attributed to the different amino acid profiles, available energy, and minerals offered by plant protein as compared to fish meal, and also to the antinutritional factors present in plant protein (such as protease inhibitors, lectins, antigenic proteins, and phenolic compounds) that could alter the nutritional status of fish with a consequent decrease in growth. However, considering that growth is a polygenic trait, it is also important to consider the influence of genetic factors on growth in the experiments. The heritability of growth has been estimated at around 41 % for farmed fish and zebrafish, indicating an important additive genetic influence in this phenotype (Von Hertell et al. 1990; Tave 1993). In zebrafish, we have observed large variations in growth among families fed a plant protein-based diet (Ulloa et al. 2013). These differences were also observed at transcriptional levels where two factors, plant protein and genetic components underlying family differences, separately modulated the expression of muscle growth-related genes in adult zebrafish (Ulloa et al. 2013). In Atlantic salmon similar factors, genotype and vegetable oil incorporated to the diet, produced different gene expression patterns in nutritional metabolic pathways (Morais et al. 2011). These results showed that certain families or genotypes respond differently to plant proteins in the diet. Therefore, nutrigenetics can be useful to investigate the specific genetic variants that influence the organism's response to nutrients (Mutch et al. 2005). Salem et al. (2012) identified SNP markers in rainbow trout under genetic selection for growth and found 23 SNP associated to growth in fish fed a commercial fishmeal-based diet. However, similar studies to identify SNP markers in genes modulated by plant source have not been carried out.

In this study, we have developed an innovative approach using RNA-Seq to identify both differentially expressed genes among fish from both tails of the weight gain distribution and select SNP from these genes to be used in a growth association study. RNA-Seq has been used independently in various studies to analyze the complete transcriptome and identifying SNP in livestock species and important commercial fish (Cánovas et al. 2013; Wickramasinghe et al. 2014; Salem et al. 2012; Liu et al. 2014a, b; Cui et al. 2014a, b; Houston et al. 2014; Qian et al. 2014). For example, in rainbow trout RNA-Seq was used to identify transcripts which are differentially expressed in the liver in response to handling and confinement stress (Liu et al. 2014a, b). In Japanese pufferfish (*Takifugu rubripens*), RNA-Seq was used to analyze global gene expression (Cui et al. 2014a) and discover SNPs from expressed genes in the swim bladder (Cui et al. 2014b). The result showed a total of 62,270 putative SNPs, and the validation revealed that 54 % of the SNPs (26/48) were real (Cui et al. 2014b). On the other hand, Houston et al. (2014) developed a dense SNP array of >400 K putative SNPs using RNA-Seq in Atlantic salmon. The analysis revealed that SNP array can distinguish between fish of different origins within and between farmed and wild populations. RNA-Seq technology can be applied to some extent in fish and represents a useful platform for high-resolution genetics research for traits economic importance in fish (Qian et al. 2014). In this study, zebrafish (*Danio rerio*) was used due their potential use in nutritional genomics studies (Ulloa et al. 2011). Zebrafish provides a useful model organism due to their short generation interval, small size, and ability to eat a wide variety of diets (Ulloa et al. 2013, 2014; Hedrera et al. 2013). In addition, zebrafish has a complete annotated genome sequence (Zv9), which facilitates performing RNA-Seq (Long et al. 2013).

Material and Methods

Fish Rearing and Diet

A population of 24 families was generated from nonrelated single-pair mates from two wild-type strains. The rearing period was split into three phases (egg production, larval period, and juvenile to adult stage) as described by Ulloa et al. (2013). Briefly, embryonic eggs were incubated at 28±1 °C until hatching in 9-cm diameter Petri dishes (Brand et al. 2002). At 7 days post-fertilization (dpf), each family was reared separately in an aerated 4 L container with a density of 25 cm3*fish^{-1}. During this period, the larvae were fed ad

libitum three times daily with commercial Sera Micron® fine powdered feed. In juvenile stage (35 dpf), 40 fish selected randomly from each family were distributed into 24 tanks. Each group of fish per family was placed in individual 14 L tanks (13×22×48.5 cm). The fish were kept at a density of 350 cm^3 $fish^{-1}$ and fed a balanced plant protein-based diet (mix of soy protein concentrate (27 %), corn (15 %) and wheat gluten meal (35 %), raw starch (7.5 %), fish oil (8.5 %), vitamin/mineral premix (4 %), choline chloride (0.5 %), and $Ca(H_2PO_4)_2$ (2 %)) until adult stages (98 dpf). In addition, the diet was supplemented with lysine amino acids (0.5 %) to fulfill the essential amino acid requirements of cyprinid fish (NRC 1993). The composition of the experimental diet was 95.2 % dry matter, 57.8 % digestible protein, 7.1 % digestible lipids, 22.5 % starch, 6.2 % ash, and 391 cal kg-1 gross energy. During all periods, the fish were reared under optimal physical and chemical parameters for water (25≤T °C≤28.5, 7≤pH≤8; hardness>100 mg CaCO3 L^{-1} and a photoperiod of 14 h light:10 h dark) (Brand et al. 2002).

Growth Measurement and Muscle Samples

At 28 dpf, a sample of approximately 20 % of the fish (n~25 fish per family) was used to record initial weight. At the end of the experiment (98 dpf), the weight was measured in all fish per family (total n=960). Weight gain was calculated as the difference between the two recording stages (WG [mg]=weight at 98 dpf−initial weight at 28 dpf). The fish were fasted for 24 h prior to each sampling event and weighed using a scale to the nearest 0.001 g (Acculab VI-3 mg). All fish were sacrificed and sexed. Muscle samples of each fish were placed in a labeled 1.5-ml polypropylene tube containing RNA-later solution and stored at minus 80 °C until RNA extraction. In order to avoid potential confounding effects in transcriptome analysis between genders, only males were considered in this study. 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.

Selection of Fish for RNA Sequencing

Fish were selected for RNA-Seq according to weight gain (WG, mg) at 98 dpf. From 440 males, 5 % from both tails of the normal distribution for weight gain were selected. These groups corresponded to 22 low-growth fish (average weight=52 mg, SD=8.03) and 22 high-growth fish (average weight=228 mg, SD=25.45). From these fish, 8 low-growth (average weight=53 mg, SD=7.00) and 8 high-growth fish (average weight=240 mg, SD=22.40) were selected for libraries preparation. These fish were chosen from different families in order to maximize genetic variation in samples within each phenotype. Two libraries for each phenotype (using 4 fish per RNA pool/library) were constructed in order to evaluate the reliability of libraries within each phenotype (Fig. 1).

RNA-Sequencing Library Preparation

Total RNA was extracted from the muscle of individual fish. The tissue was homogenized in TRIzol® (Invitrogen) following the manufacturer's protocol. RNA was quantified using a ND-1000 spectrophotometer (Fisher Thermo, Wilmington, MA), and quality and integrity were assessed according to 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. The libraries were made using an Ovation RNA-Seq System kit (NuGEN). Total RNA (100 ng) from each library was transformed to double-stranded cDNA with a median length of 200 bases and posterior SPIA (Single Primer Isothermal Amplification), followed by end repair and adaptor ligation. The Ovation RNA-Seq system performs a single round of amplification. The fragments were purified and short sequence reads of 40 base pairs (SR40) sequenced at the UC Davis Genome Center DNA Technologies Core Facility using the Illumina Genome Analyzer (GAII).

RNA Sequencing and Data Analysis

SR40 were mapped to the annotated zebrafish reference genome Zv9 (http://www.ensembl.org/index.html) using CLC Genomics workbench 3.7 (CLC Bio, Aarhus, Denmark, http://www.clcbio.com/). CLC Genomics Workbench is a multi-platform which can visualize and analyze data from next generation sequence platforms. CLC incorporates proprietary internal algorithms. Based on an annotated reference genome and mRNA sequencing reads, CLC is able to calculate gene expression levels based on the approach from Mortazavi et al. (2008). The statistics are based on RPKM (reads per kilobase exon model per million mapped reads, RPKM = total exon read/mapped reads in millions x exon length in kb). Briefly, all reads are mapped to all known genes in the chromosomes. In the first round, all uniquely mapped reads to the genes are counted, and all nonunique matches are distributed per ratio to the genes. The RPMK value is normalized for total exon-length and the total number of matches in an experiment. The expressed genes in each phenotype were ranked as high-expression (>100 RPKM), medium-expression (10–100 RPKM), and low-expression (0.2–10 RPKM). Sequencing reads from each phenotype were pooled to gene expression analysis. *T* test were performed in log2 transformed data to identify genes with significant differences in expression between phenotypes (p value<0.05 and FDR<0.2, and greater than±2.0-fold differences between phenotypes). These genes were examined by functional analysis using Blast2GO

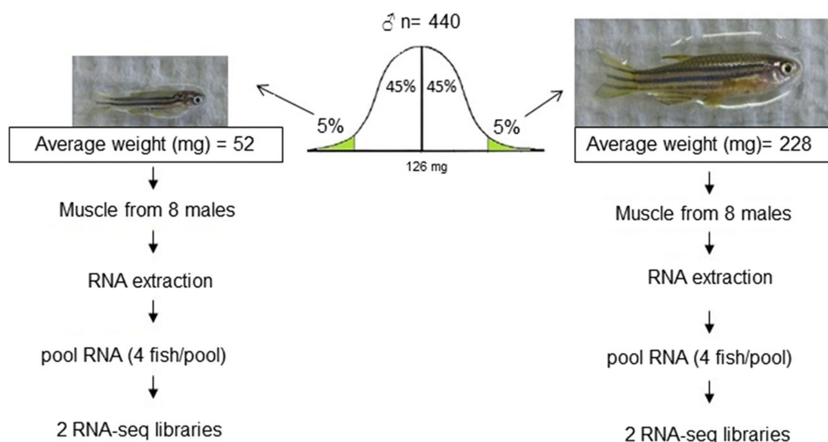

Fig. 1 Diagram showing fish selection for RNA-Seq. From a total of 440 males, 22 low-growth (average weight=52 mg) and 22 high-growth fish (average weight=228 mg) were selected from both tails of the normal distribution for weight gain (5 % of fish). Total RNA was extracted from muscle of individual fish, and two RNA pools using 4 fish per pool were used to prepare RNA-Seq libraries for both phenotypes

(Conesa et al. 2005) and submitted to bioinformatics pathway analysis (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com). These genes were choosing as candidates to identify SNP to use in a growth association study (Fig. 2).

SNP Discovery and Genotyping

SNP were detected using strict criteria and quality filters to minimize the rate of false-positive SNP detection as described by Cánovas et al. (2010). Briefly, (1) the minimum average quality of surrounding bases was set as 15 quality score units and minimum quality of the central base was set as 20 units; (2) ten reads was set as minimum coverage; (3) minimum variant frequency or count was set at 20 % per SNP; and (4) SNP located in the last three bases were not considered in the analysis. In order to select SNP from genes differentially expressed between phenotypes, the SNP completely fixed in the high or low growth group were selected. Difference in allelic frequency at each loci for all SNP selected were at least 60:40. In order to select a reduced and informative number of SNP we performed a linkage disequilibrium (LD) analysis

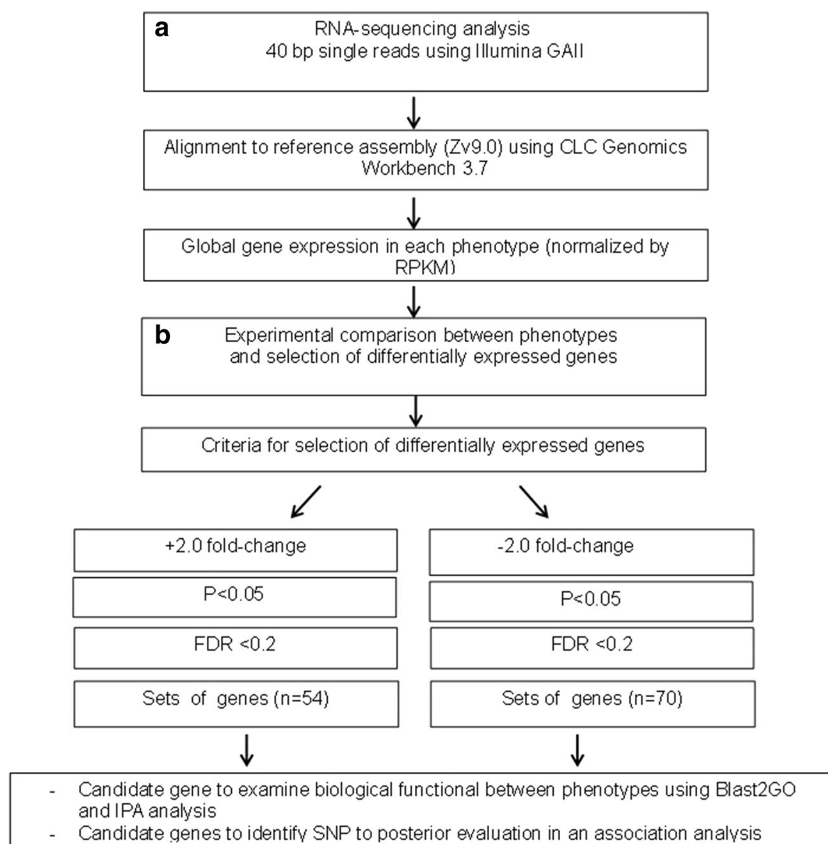

Fig. 2 Analytical flowchart for the study of transcriptome differences between low- and high-growth fish using RNA-Seq. **a** RNA-Seq analysis: Alignment to reference assembly (Zv9.0), Global gene expression in each phenotype (normalized by RPKM). **b** Experimental comparison between phenotypes and selection of differentially expressed genes (criteria ±2.0 fold changes, p value<0.05, FDR <0.2). One set of 54 genes was up-regulated in low-growth fish. Another set of 70 genes was up-regulated in high-growth fish. These genes were candidates to examine biological function among phenotypes using the Blast2GO program and submitted to IPA analysis. These genes were also candidates to identify SNP for posterior evaluation in an association analysis

using SVS7 LD module. We removed from further analysis any SNP that were in LD with each other (defined as $r2 \geq 0.9$), retaining 1 SNP with high correlation from each set. The term r2 is used to describe the correlation between SNP and to define the level of linkage disequilibrium between markers. The selected SNP were used to genotyped in 240 individuals to perform a growth association analysis and to observe the allelic distribution between phenotypes. The 240 genotyped individuals corresponded to 96 low-growth (4 fish per families) and 96 high-growth individuals (4 fish per families) and the 48 parents within the populations. Genotyping was performed using a Sequenom MassARRAY spectrometer at GeneSeek Inc., Lincoln, NE.

Growth Association Analysis

Marker-trait association analysis was performed under an additive model (Rincon et al. 2012), using the genotype association and regression modules from SNP Variation Suite (SVS) Version 7 (Golden Helix Inc., Bozeman MT, USA) (Rincon et al. 2009a, b). An *F* test was performed to find significant associations, and a *p* value threshold of 0.05 was established. False discovery rate (FDR) was controlled according to the method described by Storey (2002), and a cut-off for significant association values was set at FDR q value<0.2. We corrected for stratification and batch effects (relationship between families and parents samples) using the Principal Components Analysis (PCA) module included in SVS7 (Price et al. 2006). In order to observe the allele distribution between phenotypes, a chi-squared test with Yates' continuity correction was performed using the informative genotypes.

Results and Discussion

Global Analysis of RNA-Sequencing

RNA sequencing produced an average of 30–35 million reads for each phenotype. The total expressed genes in fish muscle were on average 17,227, about 60 % in relation to all recorded genes (28,401 annotated genes in zebrafish). This analysis defined 307 genes with high expression in low-growth fish vs. 299 genes with high expression in high-growth fish; 2241 genes with medium expression in low-growth fish vs. 2142 genes with medium expression in high-growth fish; and 14,927 genes with low expression in low-growth fish vs. 14, 538 with low expression in high-growth fish (Table 1).

In this study, two RNA-Seq libraries replication were constructed per phenotype using a pool of 4 fish for each library. This meant have a replicate with a different biological sample per library but with similar phenotype. For each phenotype a correlation analysis between replicate libraries was performed. The results revealed a correlation on average of 0.928 for highly expressed genes (>100 RPKM), 0.782 for medium expressed genes (10–100 RPKM), and 0.794 to lowly expressed (0.2–10 RPKM) indicating a good reliability to include the libraries as biological replicates. On the other hand, the selection of differentially expressed genes was made based on significant differences between phenotype (p value<0.05 and FDR<0.2, and greater than ±2.0-fold differences).

Table 1 RNA-Seq gene expression results for low-growth and high-growth fish

Category[a]	RPKM	Low-growth	High-growth
High-expression genes	>100	307	299
Medium-expression genes	10–100	2241	2142
Low-expression genes	0.2–10	14,927	14,538
Total expressed genes		17,475	16,979

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

Genes Expressed Differentially Between Phenotypes

A total of 124 genes were differentially expressed between phenotypes. One set of genes corresponds to a list of 54 genes up-regulated in low-growth compared to high-growth fish, and a second set corresponds to a list of 70 genes up-regulated in high-growth compared to low-growth fish. For both lists, the gene symbol, description, ID, *p* value, FDR, average of RPKM, and level of expression are presented in Supplementary Files 1 and 2, respectively. Among the list of 54 genes up-regulated in low-growth fish, 20 correspond to high-expression (>100 RPKM) and 34 to medium-expression genes (10–100 RPKM). Among the 70 up-regulated genes in high-growth fish, 20 correspond to high-expression (>100 RPKM), 47 to medium-expression (10–100 RPKM), and 3 to low-expression genes (0.2–10 RPKM). In order to determine the biological processes in which these genes are involved, the most specific GO term were identified, by doing an enrichment analysis using the Blas2GO program (Conesa et al. 2005). The enrichment analysis is a data mining approach to identify the functional classes that statistically differ between two lists of genes (Conesa and Götz 2008). In our case, the two lists of genes corresponded to 54 genes up-regulated in low-growth, and 70 genes up-regulated in high-growth. The results contain GO terms which were ranked according to their significances. Three different significance parameters were given such as false discovery rate (FDR), family-wise error rate (FWER), and single test *p* value (Fisher *p* value). With an FDR significance threshold of 0.05, it is possible to obtain those functionalities that are strongly significant (Conesa and Götz 2008). The result showed that differentially expressed genes are involved in totally different biological processes in the two

phenotypes. Among the 54 genes up-regulated in low-growth fish, 42 % participate in multicellular organism development (GO:0007275, FDR=4.7E^{-2}), 16 % in cell development (GO:0048468, FDR=4.7E^{-2}), 16 % in anatomical structure formation involved in morphogenesis (GO:0048646, FDR=4.7E^{-2}), 49 % in biological regulation (GO:0065007, FDR=2.2E^{-2}), 30 % in cellular component organization (GO:0016043, FDR=2.1E^{-2}), 21 % in RNA metabolic processes (GO:0016070, FDR=9.5E^{-3}), and 28 % in translation (GO:0006412, FDR=9.4E^{-4}) (Table 2). In contrast, among the 70 genes up-regulated in high-growth fish, 20 % participate in biological processes such as a sterol biosynthetic process (GO:0016126, FDR=3.6E^{-2}), 22 % in cholesterol metabolic processes (GO:0008203, FDR=2.2E^{-2}), 25 % in generation of precursor metabolites and energy (GO:0006091, FDR=7.3E^{-3}), and 42 % in oxidation reduction (GO:0055114, FDR=4.3E^{-5}) (Table 3). These biological processes could be related to the growth differences between phenotypes in response to plant-protein diets. Most of the genes up-regulated in low-growth fish are involved in biological processes related to the proliferation and differentiation of myogenic progenitor cells which participate in myoblast production and fusion, modulating fiber maturation and hypertrophy involved in growth (Weatherley and Gill 1987). Whereas that the genes up-regulated in high-growth fish are involved in biological processes related to the energy destined to synthesis of chemical reactions involved in lipid and steroids hormones, substances from which energy is derived to complete sexual maturation (Weatherley and Gill 1987). Additionally, Ingenuity Pathways Analysis also revealed different biological pathways involved in low and high-growth fish. The list of 70 genes up-regulated in high-growth fish showed a clear pattern of enrichment in the cholesterol biosynthesis pathways (p value=3.84E^{-25}). While the 54 genes up-regulated in low-growth fish are involved in other pathways as well as the eIF2 signaling and hepatic fibrosis/hepatic cell activation, but these genes do not showed a clear pattern to highly enrich these pathways (p value=7.03E^{-09}). These analyses suggest that abiotic factors, like protein sources incorporated into the feed, affect growth development in certain fish in different ways, and suggest that there are certain genotypes that are more readily adapted to a plant-protein diet. Considering that these 124 genes are differentially expressed between phenotypes and participate in different growth-related biological processes, we considered them to be good candidates for identifying potential SNP to be used in a growth association study.

SNP Selection and Growth Association Analysis

A total of 335 SNP were identified from the 124 differentially expressed genes. However, only 164 SNP were genotyped in 240 fish samples because the other SNP were in linkage disequilibrium in their respective genes. Eighty SNP were selected from 54 genes up-regulated in low-growth fish, and 84 SNP from 70 genes up-regulated in high-growth fish. These SNP were submitted to the Single Nucleotide Polymorphism Database (dbSNP) of Nucleotide Sequence Variation from NCBI and a number ID are currently available. SNP are presented in Supplementary Files 3 and 4, respectively.

Growth association analysis revealed 5 SNP associated with growth in key metabolic pathway genes, asparaginyl-tRNA synthetase (*Nars*), leiomodin2b (*Lmod2b*), CUB and zona pellucida-like domains 1 (*Cuzd1*), actin alpha skeletal muscle (*Acta1b*), and a novel protein with a PLAC8 family domain (*Plac8l1*) (Table 4). These genes have not been previously described as genes involved in fish growth. Among the 5 SNP associated with growth, statistical analysis revealed that two genes, *nars* and *lmod2b*, had SNP with significant differences in distribution of the minor alleles between

Table 2 Biological processes of 54 genes up-regulated in low-growth fish

GO term	GO number	Gene symbol	Number of genes
Multicellular organismal development	GO:0007275	myhz2, acta1b, acta1a, zgc:101853, odc1, actc1b, tnc, col12a1, nefm, pdlim7, col11a2, mdka, rps6, nap1l1, rpl6, rps29, POSTN, ucma	18
Cell development	GO:0048468	acta1b, acta1a, odc1, tnc, nefm, moka, rps6	7
Anatomical structure formation involved in morphogenesis	GO:0048646	myhz2, acta1b, acta1a, zgc:101853, pdlim7, moka, rps29	7
Biological regulation	GO:0065007	acta1b, acta1a, zgc:101853, odc1, actc1b, tnc, actn3a, cct4, nars, nefm, pdlim7, mdka, rps6, ryr3, nap1l1, rpl13, eprs, rps29, ucma, klhl31, gnb2l1	21
Cellular component organization	GO:0016043	rps6, acta1b, acta1a, actn3a, col11a2, col12a1, eprs, pdlim7, tnc, moka, nap1l1, nefm, si:ch211-267e7.7	13
RNA metabolic process	GO:0016070	rps6, nop10, nars, dnajc8,, nap1l1, eprs, AL935186.6, BX296557.6, zgc:101853	9
Translation	GO:0006412	rpl18a, rpl15, rps6, rpl10, rpl13, rpl6, rps29, eprs, eef2b, nars, fau, zgc:101853	12

Table 3 Biological processes of 70 genes up-regulated in high-growth fish

Go term	GO number	Gene symbol	Number of genes
Sterol biosynthetic process	GO:0016126	ebp, sc5dl, sc4mol, dhcr24, nsdhl, hsd17b7, dhcr7, hmgcs1, fdps, cyp51, sqle, fdft1, cyp7a1a, hsd3b7, fdx1, hmgcra	16
Cholesterol metabolic process	GO:0008203	ebp, sc5dl, dhcr24, nsdhl, hsd17b7, dhcr7, hmgcs1, fdps, acat2, cyp51, sqle, fdft1, cyp7a1a, hmgcra	14
Generation of precursor metabolites and energy	GO:0006091	uqcrfs1, fdx1, idh1, atp5ia, mt-cyb, mt-nd4, ndufa1, slc25a14, ndufa10, mt-nd1, aco2, mt-nd5, uqcrc1, mt-co2, mt-co1, mt-nd2	16
Oxidation reduction	GO:0055114	sc5dl, sc4mol, dhcr24, nsdhl, hsd17b7, dhcr7, cyp51, sqle, fdft1, cyp7a1a, hsd3b7, uqcrfs1, si:dkey-91i10.3, fdx1, idh1, hmgcra, mt-cyb, BX927387.2, mt-nd4, ndufa1, ndufa10, mt-nd1, mt-nd5, uqcrc1, mt-co2, mt-co1, mt-nd2	27

phenotypes (Table 5). *Nars* is involved in cellular movement and development and participates in aminoacyl-tRNA biosynthesis pathways. Aminoacyl-tRNA is a substrate for translation and is pivotal in determining how the genetic code is interpreted as amino acids (Ibba and Söll 2000). In *Nars* (transcript ID ENSDART00000086746 with 2462 bp), 3 SNP were genotyped in 240 samples. Two SNP were located in the 3′-UTR site (rs318240509, rs318240510) and the third one on exon 13 (rs318240508) (Fig. 3). The result revealed that only one synonymous SNP (A/T, rs318240508) (Table 4) was associated with the growth phenotype (*p* value 0.0001 and FDR 0.001), where the frequency of the minor allele (T) was 0.129. On the other hand, the statistical analysis revealed a significant difference of the minor allele distribution between phenotypes (Table 5). The presence of the minor allele was more frequent in low-growth compared to high-growth fish (0.18 vs. 0.01) (x^2=21.42; *p* value=3.6×10−6) (Table 5). Another gene, *Lmod2b*, encodes a tropomyosin-binding actin protein enriched in the skeletal muscles (Takebayashi et al. 2009). This protein contributes to the final organization and maintenance of sarcomere architecture by promoting tropomyosin-dependent actin filament (Skwarek-Maruszewska et al. 2010). In this gene (transcript ID ENSDART00000067409 with 3231 bp), 2 SNP (rs318240392, rs318240393) located in exon 2 (at 44 and 762 position, respectively) were genotyped (Fig. 3). The association analysis revealed that only one SNP (G/C, rs318240392) was associated with growth (*p* value 0.0061 and FDR 0.158), where the frequency of the minor allele (C) was 0.223 (Table 4). This variation corresponds to an amino acid substitution in the protein (Ser141Thr) (Table 4). According to the SIFT tool (http://sift.bii.a-star.edu.sg/), which predicts if a mutation is deleterious on protein function or not, this change showed a score of 0.39 which indicate that the mutation could be tolerated. The allelic distribution showed that the presence of the minor allele (C) was significantly more frequent in high-growth compared to low-growth fish (0.31 vs. 0.12) (x^2=11.39; *p* value=0.00073) (Table 5). Considering the heterogeneity in the distribution of the minor allele between phenotypes in two SNP (rs318240508 present in *nars* and rs318240392 present in *lmod2b*), the results suggest that these SNP could shed light on which zebrafish individuals are the more or less efficient for growth when fed a plant-protein diet.

Another three genes (*Cuzd1*, *acta1b*, *plac8l1*) also had SNP associated with growth (Table 4). However, in terms of allelic distribution, statistical analysis showed that the distribution of the minor alleles was not significantly different between phenotypes (*p* value>0.001) (Table 5). The *cuzd1* gene encodes a transmembrane-associated protein, involved in diverse functions including developmental patterning, tissue repair, cell signaling, inflammation, receptor-mediated endocytosis, and tumor suppression (Abdul Ajees et al. 2006; Perry et al. 2007). In this gene (transcript ID ENSDART00000128412

Table 4 SNP associated with growth phenotype in zebrafish

Gene	Gene information ID gene and SNP position	SNP ID	SNP	Minor allele	Minor allele frequency	*p* value	FDR	Amino acid
Nars	ENSDARG00000061100_17741	rs318240508	A/T	T	0.129	0.0001	0.001	Synonym
Lmod2b	ENSDARG00000045864_1034	rs318240392	G/C	C	0.223	0.0061	0.158	Ser141Thr
Cuzd1	ENSDARG00000089599_4308	rs318240447	A/C	C	0.031	0.0056	0.173	Ile500Leu
Acta1b	ENSDARG00000055618_5089	rs179423287	C/T	T	0.200	0.0033	0.172	–
Plac8l1	ENSDARG00000087764_4071	rs318240487	T/A	A	0.132	0.0050	0.195	–

Table 5 Frequency of minor alleles between phenotypes present in each SNP associated with growth

Gene	SNP ID	Allelic frequency between phenotypes (minor allele represented by d)						Significance
		Low-growth fish			High-growth fish			
		N	d	D	N	d	D	
Nars	rs318240508	76	0.18	0.82	64	0.01	0.99	3.6×10−6 ***
Lmod2b	rs318240392	73	0.12	0.88	43	0.31	0.69	0.00073 ***
Cuzd1	rs318240447	71	0.02	0.98	72	0.00	1.00	0.1199 ns
Acta1b	rs179423287	85	0.16	0.84	76	0.20	0.80	0.3868 ns
Plac8l1	rs318240487	78	0.10	0.90	79	0.14	0.86	0.3040 ns

***Indicates significant difference, p value<0.001

N Number of informative genotype used to observe the allele distribution in each phenotype, *ns* no significant difference, *d* minor allele, *D* major allele

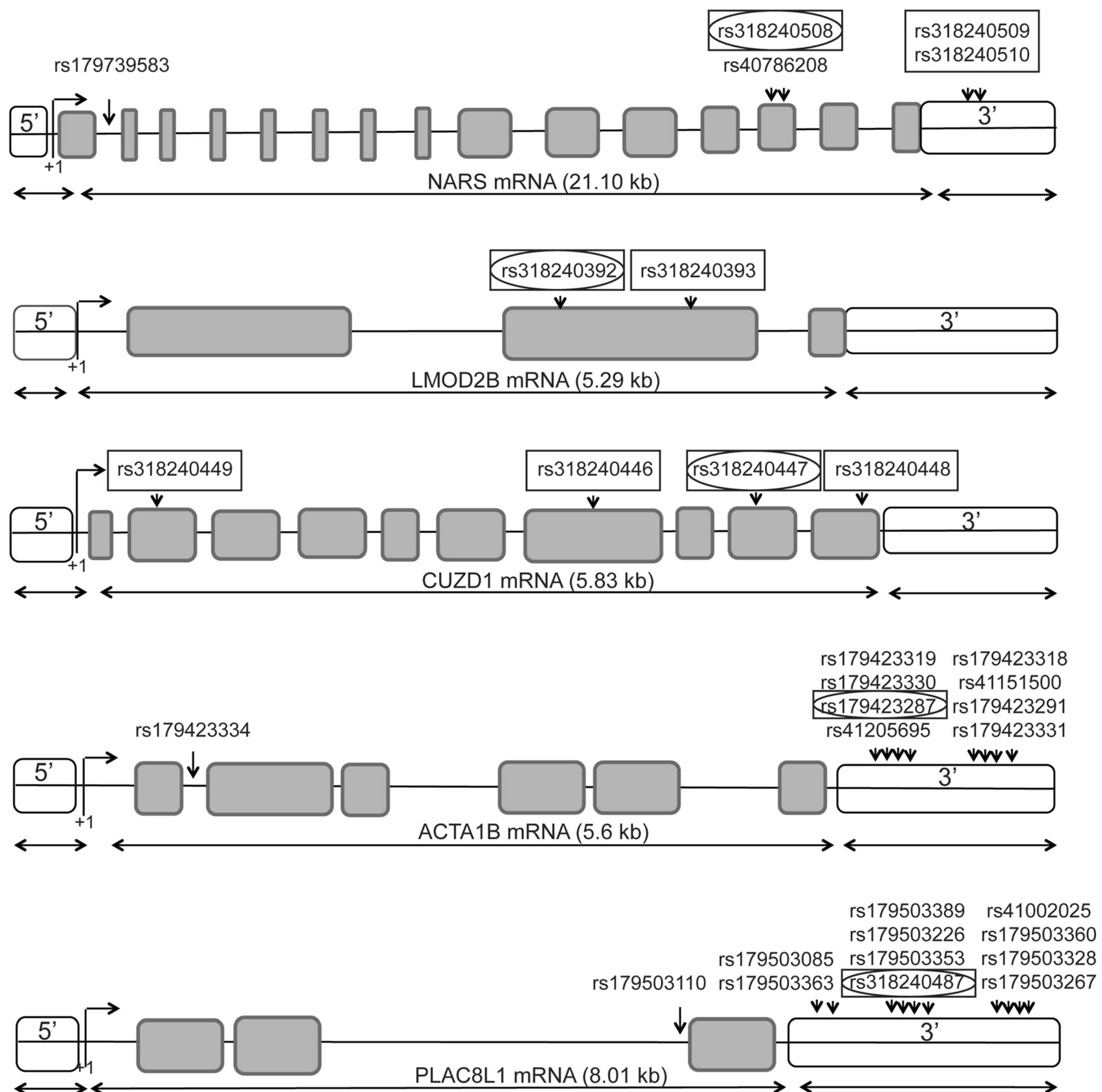

Fig. 3 Localization of SNP associated with growth in the promoter and coding region of asparaginyl-tRNA synthetase (NARS); leiomodin2b (LMOD2B), CUB and zona pellucida-like domains 1 (CUZD1); actin alpha skeletal muscle (ACTA1B) and novel protein with a PLAC8 family domain (PlAC8L1). Exon/intron distribution is taken from the zebrafish gene. In *square*: SNP genotyped in 240 samples. In *circle*: SNP significantly associated with growth in zebrafish

with 2735 bp), 4 SNP (rs318240449, rs318240446, rs318240447, and rs318240448) located at exon 2, 7, 9, and 10, respectively, were genotyped (Fig. 3). The results showed that only one SNP (A/C, rs318240447), located on exon 9, was associated with the growth phenotype (*p* value=0.0056 and FDR=0.173), where the frequency of minor allele (C) was 0.031 (Table 4). This variation corresponds to an amino acid substitution in the transmembrane-associated protein (Ile500Leu), and the predicted effect on protein function according to SIFT was 0.1 (Table 4). Another two genes (*Acta1b* and *Plac8l1*) also had SNP located in the 3-UTR region associated with growth. *Acta1b* encodes the α-actin isoform in adult skeletal muscle, which participates in the formation of the thin filament of sarcomere where it interacts with a wide array of proteins, notably myosin, to produce muscle contractions (Laing et al. 2009). In this gene (transcript ID ENSDART00000058629 with 1542 bp), only one SNP (C/T, rs179423287) was genotyped (Fig. 3) and associated with growth (*p* value=0.0033 and FDR=0.172), where the frequency of the minor allele (T) was 0.20 (Table 4). Another gene, *plac8l1*, encodes a small cysteine-rich protein conserved in vertebrates. In mice, this protein has been suggested to have a role in innate immune function as an intracellular protein required for optimal function of the neutrophils to kill bacteria (Ledford et al. 2007). It has also been shown that *plac8* is an upstream regulator of C/EBPb, which is required for normal differentiation of adipose tissue, as well as for resistance to obesity (Jimenez-Preitner et al. 2011). In this gene (transcript ENSDART00000128778 with 1514 bp), only one SNP (T/A, rs318240487), located at 3-UTR, was genotyped (Fig. 3) and associated with growth phenotype (*p* value=0.0050 and FDR=0.195), where the frequency of the minor allele (A) was 0.132 (Table 4).

At the molecular levels, SNP located in 3′-UTR could shed light on the effect of mRNA structure and stability, and SNP located in exon regions can affect the function of proteins (Mignone et al. 2002). All of these effects could influence fish growth response. However, considering that growth is a complex trait controlled by hundreds of genes and QTL regions, these markers also could be in linkage disequilibrium with other genetic polymorphisms. For example, in *acta1b* the sequence flanking the variant location ~1000 bp upstream and downstream showed at least 12 neighboring variants in intronic regions and 7 variant in 3′- UTR regions. The gene *plac8l1* showed 9 neighboring variants in 3′-UTR regions. In contrast, the genes *nars*, *cuzd1*, and *lmod2b* have synonymous variants. Each of these neighboring variants could also contribute to a minor effect on growth phenotype. Few QTL related to growth have been identified in zebrafish. The only study describing QTL in zebrafish showed a QTL for growth rate and muscle fat content on chromosome 23 (Wright et al. 2006). However, none of the genes presented in this study (*cuzd1*, *acta1b*, *plac8*, *lmod2b*, and *nars*) are located in chromosome 23. *Nars* is located at chromosome 21, where a QTL for antipredator behavior has been described in zebrafish (Wright et al. 2006). Unlike in zebrafish, many QTL for length and weight have been described in farmed fish like Atlantic salmon (Baranski et al. 2010), rainbow trout (Wringe et al. 2010), and Arctic charr (*Salvelinus alpinus*) (Küttner et al. 2011). Even QTL for growth and morphometric traits have been detected in channel catfish (*Ictalurus punctatus*)×blue catfish (*Ictalurus furcatus*). The results showed that at least 11 of 44 linkage groups had one significant QTL for measured traits (Hutson et al. 2014). Some studies have detected synteny of QTL related to growth among commercial and model fish (Wringe et al. 2010; Küttner et al. 2011). For example, Küttner et al. (2011) reported some conservation of QTL involved in body weight and condition factor among rainbow trout, Arctic charr, and Atlantic salmon. Wringe et al. (2010) reported a comparative synteny of the rainbow trout QTL regions for body weight to their putative homologous chromosomal segments in medaka and sticklebacks. These authors indicated that a significantly greater proportion of approximately 100 selected candidate genes (influencing metabolism and growth) in medaka and stickleback were homologous to rainbow trout QTL (Wringe et al. 2010). Moreover, synteny among zebrafish and European sea bass (*Dicentrarchus labrax*) (Guyon et al. 2010), Japanese flounder (Castano-Sánchez et al. 2010), turbot (*Scophthalmus maximus*) (Bouza et al. 2012), grass carp (*Ctenopharyngodon idella*) (Xia et al. 2010) and channel catfish (*Ictalurus punctatus*) (Jiang et al. 2013) have also been detected. For example, Jiang et al. (2013) conducted a whole genome comparative analysis between channel catfish and four model fish species, zebrafish, medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*) and green spotted pufferfish (*Tetraodon nigriviridis*). The results indicated that based on the location of homologous genes, homologous chromosomes were determined among catfish and the four model fish species. A large number of conserved syntenic blocks were identified between catfish and the four model fishes. However, the analysis supported that the catfish genome is most similar to the genome of zebrafish. Therefore, considering the synteny between farmed and model fish, the genes reported in this study could be good candidates for further study in farmed fish using comparative genomics. These genes could be evaluated for potential SNP associated with growth in farmed fish fed a plant protein-based diet.

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Conflict of Interest The authors declare that they have no competing interests.

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