Research Article

Comparison of gene expression patterns of key growth genes between different rate growths in zebrafish (*Danio rerio*) siblings

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ABSTRACT. Variable individual growth rate is a phenomenon observed in fish cohorts that influences the aquaculture performance and fish cohort ecological viability. Our aim was to compare gene expression patterns of key growth genes in zebrafish larvae with different growth rate. The body length of sibling zebrafish larvae at 6 days post hatching (dph) was measured. The larvae were reared to 20 dph and measured again. Two body-length groups were clearly observed: 4 mm (small larvae) and 5-6 mm (large larvae). Total RNA was isolated from both groups. Growth hormone (gh), growth hormone receptor (ghr), insulin-like growth factor 1 (igf-1), insulin-like growth factor receptor (igf-1r a/b), insulin-like growth factor binding protein 1 (igfbp-1), thyroglobulin (tg), cholecystokinin (cck), and ghrelin were evaluated by quantitative polymerase chain reaction (qPCR). Glucokinase (gck) and igfbp-1 were included as a gene expression marker of larvae nutritional status. Two genes showed significant differences between the body length groups, igfbp-1 (P = 0.01) and igf-1r (P = 0.02). The igfbp-1 suggests than growth rate variability was associated with the larvae nutritional status and this condition affect the gene expression pattern of igf-1r. Therefore these genes are interesting genes markers for growth rate variabilities studies.

Keywords: *Danio rerio*, zebrafish, growth rate, IGF-1R, IGFBP-1, aquaculture.

INTRODUCTION

Fish larvae stage is a developmental phase after the embryogenesis, in which the metamorphosis process generates a major morphological and physiological changes intrinsically associated with each species of fish (Dufour et al., 2012). From a point of view of cohort growth, at hatching the larvae showed similar body length, however within a short period of time is possible observed size heterogeneity among individuals; due a natural phenomenon called growth rate variability (Deangelis & Coutant, 1979; Kestemont et al., 2003). Growth rate variability is a natural phenomenon, which influencing individual performance either from an ecological perspective (Pepin et al., 2015) or in a fish aquaculture productivity (Goldan et al., 1997; Lekang, 2013). In fish farming, size heterogeneity is not ideal, because the subordinate fish have less access to the feed, have more stress and increase the possibility of cannibalism (Lekang, 2013). Size heterogeneity in the cohorts are expressed mathematically as the coefficient of variation (CV) (Weiner & Solbrig, 1984).

The physiological process of growing is mainly regulated by the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis (Reinecke et al., 2005). Which is a pleiotropic physiological axis of hormones and cellular receptors that regulate: nutrients metabolism, protein synthesis in muscle and in general tissue growth and osmotic balance (Butler & Le Roith, 2001; Reinecke et al., 2005). The endocrine activity of GH has two pathways: the direct, in which the physiological effects are mediated by GH binding to its receptor (GHR), and indirect pathway, in which GH induces IGF-1 secretion and promotes biological activities (Canosa et al., 2007). Among insulin growth factors: IGF-1, IGF-2 and IGF-3; IGF-1 is the main hormone in the regulation of larvae growth, because IGF-2 expression is only observed at embryogenesis (Wood et al., 2005) and IGF-3 is gonad specific (Wang et al., 2008). How a counterpart of hormones are the growth axis receptors: the GHR is a single transmembrane glycoprotein that belongs to the class I cytokine receptor superfamily (Pérez-Sánchez et al., 2002), and the IGF-1 receptor belongs to the tyrosine kinase superfamily of transmembrane receptor like the...
insulin (Wood et al., 2005). On the other hand, insulin-like growth factor binding protein (IGFBP) is a protein family of six members can bind IGF-1 and IGF-2, their roles are to increase the half-life of IGFs and distribution, which also are been described in fish (Daza et al., 2011; Reindl & Sheridan, 2012), the most abundantly in plasma are IGFBP-1, IGFBP-2 and IGFBP-3 (Wood et al., 2005).

Furthermore many peptides or hormones stimulating or inhibiting the GH/IGF-1 axis (Canosa et al., 2007; Chang & Wong, 2009), and affect the growth. The thyroid hormones triiodothyronine (T3) and thyroxine (T4) have stimulatory effects on GH and IGF-1, and they are the principal factors controlling metamorphosis in fish larvae (Wang & Zhang, 2011; McMenamin & Parichy, 2013). Ghrelin is a peptide hormone mainly secreted in the oxyntic mucosa of the stomach and is the ligand for the growth hormone secretagogue receptor (GHS-R); which is the other endocrine pathway that stimulates the secretion of GH by the pituitary gland in addition to the GH-releasing hormone (Dimaraki & Jaffe, 2006). Additionally, ghrelin is an orexigenic factor that increases food intake and plays an important role in energy and glucose homeostasis (Peter & Chang, 1999; Nakazato et al., 2001; Unniappan & Peter, 2005; Dimaraki & Jaffe, 2006; Arcamone et al., 2009; Pradhan et al., 2013). Cholecystokinin (CCK) is a peptide hormone secreted by the gastrointestinal tract, and its effects include gallbladder and pancreatic secretion, gastric and intestinal motor function, reduced food intake and stimulation of GH secretion (Canosa et al., 2007; Crespo et al., 2014; Micale et al., 2014; Dalmolin et al., 2015).

Many reports have observed that fasting or poor nutritional status in fish alters the mRNA expression of components of the GH/IGF-1 axis, such as: starving Oncorhynchus kisutch and Lates calcarifer (Duan & Plisetskaya, 1993; Matthews et al., 1997) or in fasting Anguilla japonica, Dicentrarchus labrax, Ictalurus punctatus and Oncorhynchus mykiss (Duan & Hirano, 1992; Norbeck et al., 2007; Terova et al., 2007; Peterson et al., 2009).

Growth rate variability has been attributed to both biotic and abiotic mechanisms, which have been categorized as either “imposed” or “inherent” (Huston & DeAngelis, 1987; Kestemont et al., 2003). Imposed mechanisms include: temperature, day length, food availability, and interactive factors such as food competition. On the other hand, inherent mechanisms have strong genetic influence, so high cohort genetic variability increases the growth rate variability (Nicieza et al., 1994; Hutchings & Jones, 1998; Ohlberger et al., 2013). Minimal information is available regarding gene expression patterns of growth factors due the growth rate variability process, as well is necessary define a main mechanism and tested in an isolated fashion. Food competition was the main mechanism proposed for this study, which may influence a poor nutritional status in some individuals; hence larvae growth rate may be associated with key growth gene expressions patterns. Our aim was assess the key growth gene patterns observed at growth rate variability process. This study was carried out in environmentally controlled conditions (for the control inherent-non interactive mechanism) and to minimize genetic variability in the zebrafish larvae cohort, we used siblings (for the control imposed genetic mechanism).

MATERIALS AND METHODS

Experimental animals

From a spawning with one pair of adult zebrafish we obtained 100 viable eggs, which were incubated at 26°C; only those larvae that hatched between 48 to 72 h after spawning were included. In the experimental design proposed, the use hatching siblings sought to reduce the cohort genetic variability. Larvae, were maintained in glass flasks with 2 L of E2 methylene blue media (Westerveld, 2000) under controlled light/dark conditions (14L/10D), with a 30% of water change every day. At 6 days post-hatching (dph) or 156 accumulated thermal units (ATU), the body lengths of co-hatched zebrafish larvae were measured. The larvae standard length measurement was conducted under stereoscopic microscope with a Motic® Images Plus 2.0ML software according to the proceeding proposed by Parichy et al. (2009); the larvae were previously anesthetized by benzocaine 20% (0.2 mL L-1). Subsequently, total RNA was isolated from 30 zebrafish larvae (4 mm body length) to establish the initial state of gene expression (reference group); these larvae were grouped into five pools or biological replicates of six larvae each. The other co-hatching siblings larvae were reared for 14 days and were fed with rotifers (Brachionus plicatilis), at the rate of 200 rotifers per larva per day (Lawrence et al., 2012). At 20 dph, the body length of each remaining larva was measured, and then 60 larvae were classified into two groups: large larvae and small larvae. The small larvae group was composed of 30 individuals with a body length of 4 mm, and they were divided into five pools or biological replicates with 6 larvae each. The large larvae group was also composed of 30 individuals divided into five pools or biological replicates of 6 larvae each. The large larvae groups were organized in its body length as follow: 4 larvae groups of 5 mm and one larvae group of 6 mm. This study was conducted in...
strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the INTA Universidad de Chile.

**Total RNA isolation and reverse transcription**

All zebrafish larvae pools (n = 15, small-large reference) were placed in 1.7 mL microcentrifuge tubes; excess liquid was removed, and euthanasia was performed by freezing at -80°C in liquid nitrogen. Total RNA of all pools was isolated using 800 µL of Tripure® reagent (Roche) (Lan et al., 2009), according to the manufacturer’s instructions. RNA was quantified with a spectrophotometry at 260 and 280 nm (Nano-Drop®) and the RNA quality was assessed with 1% agarose gel electrophoresis. They were treated with RQ1 RNase- Free DNase (cat. M6101, Promega®) to avoid genomic DNA amplification, the absence of genomic DNA was confirmed by PCR on the treated RNA. The first-strand cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega®). Total RNA was combined with 0.5 µg reaction1 Oligo(dT)15 Primer (cat. C1101, Promega®) for a final volume of 5 µL and incubated at 70°C for 5 min. Next, 15 µL of the transcription mix (ImProm-II™ 5X Reaction Buffer 4.6 µL, 2.25 mM of MgCl2, 0.5 mM each dNTP, Recombinant RNasin® Ribonuclease Inhibitor (Promega®) 20 µL and 1 µL ImProm-II™ Reverse Transcriptase (Promega®)) was added. Following the addition of transcription mix, the reaction was maintained at 25°C for 5 min and then transferred to 42°C for 60 min. The reverse transcription reactions were stopped by heating the mixture at 70°C for 15 min.

**qPCR analysis**

The gene-specific oligonucleotide primers for growth hormone (gh), growth hormone receptor (ghra), insulin-like growth factor 1a (igf-1a), insulin-like growth factor receptor a and b (igf-1r a and b), insulin-like growth factor binding protein 1 (igfbp-1), ghrelin (ghrl), cholecystokinin a (ckc) and glucokinase (gck) were developed using Primer-BLAST (NCBI) (Ye et al., 2012). To test the modulation of T3 and T4 in fish larvae, we assessed the gene expression of their precursor protein, thyroglobulin (tg). For normalization of cDNA loading, all samples were run in parallel using the housekeeping gene elongation factor I-alpha (ef1α) as the reference gene (McCurley & Callard, 2008). All primers are listed in Table 1. The relative mRNA expression levels of target genes and the reference gene (ef1α) were quantified using real-time PCR analysis with AriaMx Real-Time PCR (Agilent Technologies).

Amplification of specific PCR products was detected using the FastStart Essential DNA Green Master® (Roche), according to the manufacturer’s instructions. All cDNA examples were analyzed in duplicate. The amplification protocol used was as follows: one initial step of 10 min at 95°C (denaturation and enzyme activation), followed by 45 cycles of 95°C for 10 s, 60°C for 5 s and 72°C for 15 s. After the amplification, melting curve analysis was performed over a range of 50-95°C to verify that a single PCR product was generated at the end of the assay.

**Data & statistical analysis**

The cohort’s coefficients of variation were calculated based on the formula proposed by Sokal & Rohlf (1995):

\[ CV = \left(1 + \frac{1}{4ns}\right)(\frac{\sum x^2}{n} - \frac{\overline{x}^2 x}{n}) \]

where \( n \) is the number of observations; \( s \) is the sample standard deviation, and \( x \) is the sample mean. Density histograms were made using the program R-3.1.2 for Windows (32/64 bit) (R Core Team, 2014).

The relative expression levels of the genes were calculated by the method of Pfaffl (2001), using the reference group as a control group in the equation. The primer PCR efficiency (E) was calculated for each gene fluorescence curve with LigRegPCR 12.18 software (Udvardi et al., 2008), and the efficiency rates for the transcripts were as follows: 1.96 for gh, 1.87 for igf-1, 1.91 for tg, 1.8 for igfbp, 1.82 for igf-1r(a/b), 1.7 for gck, 1.88 cck, 1.96 ghrelin and 1.84 for ef1α over the entire quantification range. The differences in the gene expression levels were analyzed by a Wilcoxon-Mann-Whitney test (Derveaux et al., 2010) between the small and large pools using R-3.1.2 for Windows (32/64 bit) (R Core Team, 2014). P-values <0.05 were considered significant. In addition, gene expression was analyzed by principal component analysis (PCA) (Abdi & Williams, 2010). The principal component analyses were made using the FactoMineR packages and the biplot by Factoextra and ggplot2 packages in R-3.1.2 (Ringner, 2008; R Core Team, 2014).

**RESULTS**

**Body size heterogeneity**

The distributions of larval body length at 6 dph and 20 dph are presented in the (Figs. 1a-1b). The larvae body length distribution observed in the beginning of the study (6 dph), showed a mode associated with 4.0 mm in body length. This mode represented nearly 72% of the measured larvae, and the remaining larvae were
shorter than 4 mm. At 20 dph, body length distribution evolved to show two modes, 4.0 mm (46%) and 5.0 mm (44%), and 10% of the larvae were 6 mm long, representing the longest larvae. The coefficient of variation between the cohorts changed from 5.79 at 6 dph to 14.22 at 20 dph. The mortality during the rearing period was 8%.

**Modulation of genes related to growth**

In the GH/IGF-1 axis, differences in gene expression between small and large larvae are presented in Fig. 2. Only were statistically significant for the *igf-1* (a/b) receptor (Wilcoxon-Mann-Whitney test, $P = 0.02$) and *igfbp-1* (Wilcoxon-Mann-Whitney test, $P = 0.01$), the other genes of growth axis were not statistically significant ($P > 0.05$) and showed similar levels of gene expression between the groups. The expression levels of the other genes evaluated (*tg*, ghrelin, *ccka*, *gck*) were not statistically significant (Fig. 3), although for *gck*, the Wilcoxon-Mann-Whitney test yielded $P = 0.07$.

**Principal components analysis (PCA)**

This descriptive analysis revealed other aspects of variability among individuals (the larvae pools) and variables (genes), enabling us to elucidate the main components of the variability. The PCA results revealed that approximately 84.4% of the inertia was explained by four components or dimensions (PCs): PC1 = 26.6%, PC2 = 23.6%, PC3 = 21% and PC4 = 13.2%. The PCA with PC1 and PC2 (Figs. 4a-4b). Figure 4a shows the correlation among variables (gene expression). There is a positive correlation between *gck*...
and igf-1r, as well as between tg and igfbp-1. A projection in the first component (PC1) is apparent in both groups and the two projections are negatively correlated. Figure 4b shows the individuals (larvae pools) and circumscribed groups associated with larval body length. This aggregation is projected onto PC1, the large larvae are on the negative side and the small larvae on the positive side. The correlations among components and variables are presented in Table 2, which shows that the most important genes correlated with PC1 were igfbp-1 (0.86, P = 0.001), gck (-0.67, P = 0.03) and tg (0.65, P = 0.03). Therefore, gck was significantly correlated with PC1 and its projection was associated with large larvae, while tg and igfbp-1 were significantly correlated with PC1, although their projections were associated with small larvae (Figs. 4a-4b). In PC2, the most important correlations were cck (0.92, P = 0.0001) and ghr (0.66, P = 0.03).

**DISCUSSION**

The present study confirms that the growth rate variability phenomenon modulate the gene expression patterns of growth endocrine control genes. The siblings zebrafish cohort presented a growth rate variability after the rearing period, because the larval body length distribution began with low CV (5.8%) at 6 dph and was raised to 14.22% at 20 dph. The size length variation observed in the study is according to the size variation observed in other studies in *Sparus aurata* (Goldan et al., 1997) or in *Sciaenops ocellatus* (Smith & Fuiman, 2003) and this situation is consistent with the report of Kestemont et al. (2003) in the growth rate variability phenomenon.

Larvae body length groups not showed significant differences in *gh* and *igf-1* mRNA levels. However, the mean of *gh* mRNA levels was slightly higher in smaller larvae than in large larvae, this trend conforms to the expectations associated with the poor nutritional status (Wood et al., 2005; Norbeck et al., 2007; Savage, 2013). Conversely, was observed uniformity in *igf-1* mRNA levels between the larvae groups, in contrast to the most fasting reports in different fish species (Wood et al., 2005; Norbeck et al., 2007; Peterson & Waldbieser, 2009; Reinecke, 2010; Kawaguchi et al., 2013; Tian et al., 2015; Taniyama et al., 2016). However, Wen-Ying et al. (2012) in *Carassius auratus gibelio*, Breves et al. (2014) and Fox et al. (2010) in Mozambique tilapia (*Oreochromis mossambicus*), and Hevst et al. (2011) in Atlantic salmon (*Salmon salar*) only observed significant differences at the protein le-
levels but not at the mRNA levels by fasting challenge. Less information is available about the ghrelin and ghrelin-like growth factor receptor (ghr) mRNA levels modulation by fasting challenge, the fish studies showed variable results. In channel catfish (Ictalurus punctatus) by increasing feeding levels, ghr mRNA levels did not change significantly between the groups (Peterson et al., 2008). However in rainbow trout (Oncorhynchus mykiss) the fasting reduce ghr mRNA levels (Walock et al., 2014); conversely fasting increase ghra and ghrb mRNA levels in zebrafish (Danio rerio) (Tian et al., 2015). Regard to igf-1r mRNA levels the fish studies also have been showed variable results. As well, the in Ictalurus punctatus and Oncorhynchus mykiss, it did not show mRNA levels modulation by fasting (Peterson et al., 2009, Gabillard et al., 2003); though Norbeck et al. (2007) found igf-1r up-regulation in gill but not in skeletal muscle in fasting Oncorhynchus mykiss. Nevertheless, this studies analysys igf-1r mRNA levels by specific tissue, nonetheless this receptor is expressed in all body tissues (Wood et al., 2005; Nimpfch & Giovannucci, 2012); therefore, these results give a partial interpretation of the igf-1r expression. Conversely, our study used the complete larvae and the igf-1r gene expression adds all body tissues. Hence, the significant difference between the body length groups in igf-1r suggests that the gene expression of igf-1r may be more sensible by the larvae nutritional status than its main ligand igf-1.

In fish fasting challenge influence the modulation of gastrointestinal peptides as cholecystokinin and ghrelin. The cck mRNA levels decrease by fasting in different fish species (Murashita et al., 2006; Feng et al., 2012; Ji et al., 2015), conversely ghrelin increase mRNA levels by fasting (Amole & Unniappan, 2009; Zhou & Xue, 2009; Tian et al., 2015; Volkoff, 2015; Blanco et al., 2016). These gene expression modulations were not observed in our results.

The most likely explanation of the key growth gene expression patterns results could be associated to the larvae nutritional status. The igfbp-1 and gck gene expressions are regulated by nutritional status or the glucose levels. The igfbp-1 is regulated by insulin levels and in consequently with glucose levels (Lee et al., 1993) and fasting increase its mRNA levels in fish (Shimizu et al., 2006; Hevrøy et al., 2011; Kawaguchi et al., 2013; Breves et al., 2014), according to the our study results. On the other hand, gck is a liver enzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate (Enes et al., 2009), is associated with individual nutritional status and showing an up-regulation by feed intake (Caseras et al., 2000; González-Alvarez et al., 2009; Panserat et al., 2014). Likewise, the main significant correlations in PCA analysis were according with this interpretation. The large larvae could be associated with a high food intake or better nutritional status and this increase gck mRNA levels; this is according with the high significant correlation observed between these factors. On the other hand, the igfbp-1 was correlated with small larvae; this outcome was according with the igfbp-1 modulation by poor nutritional status. Hence, the results suggest that the growth rate variability was associated with the larvae nutritional status, and possibly influenced by food competition (Ruzzante,
Key growth genes modulation by growth rate variability in zebrafish larvae

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