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Exploring Transcriptomic Patterns in Slow- and Fast-growing Seriola dorsalis Larvae

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Abstract: *Seriola* species have traditionally been a major component of global commercial and recreational fisheries, and in recent years, aquaculture value of these fish has grown into a ~$1.3 billion industry. The California yellowtail (*Seriola dorsalis*) is a strong candidate for development of offshore commercial aquaculture in southern California and neighboring Baja California. Although production from broodstock populations has been successful, it has not yet reached a sustainable level where it can satisfy the aquaculture demand, largely due to difficulties from highly variable survival and growth rates through the larval stages. Given the extremely fast growth and the major physical changes that occur during the earliest life stages, one way to examine variability in survival and growth is at the gene and molecular levels across those early-developmental periods. To improve our understanding of the molecular processes underlying development, we examined RNA-Seq profiles for several early life stages of yellowtail, categorized as either slow- or fast-growing. Gene expression was measured in three replicates of pooled larval samples at 2, 7, and 17 days post hatch for these two growth categories. Using the Illumina platform, an average of sixty million reads were obtained per replicate; genes of related function were sorted into clusters, and those found at high frequency in the differential gene expression set were identified. Differences in molecular pathways, biological processes, and gene regulating patterns between the two fitness groups were examined. There were many differentially expressed genes across developmental stages and between the fitness groups. For example, genes involved in oxidative phosphorylation pathways revealed interesting patterns both across developmental stages and between slow- and fast-growing larvae.

Key words: Aquaculture, Seriola dorsalis, RNA-Seq, growth-heterogeneity, larvae

Introduction

In the United States, the native California yellowtail (*S. dorsalis*) is considered a prime candidate for aquaculture development in Southern California and is the target for an aquaculture industry poised for rapid growth in North America, with offshore net pens in place off northern Baja California, and proposals to develop similar pens off the coast of Southern California. However, the feasibility of commercial-scale culture for this and other *Seriola* species hinges on reliable juvenile production from broodstock populations. In Japan, *Seriola* culture has traditionally relied on harvesting and growout of wild juveniles, but more recently, focus has shifted to closed life-cycle production to help alleviate pressure on natural populations and to generate a more predictable supply of juveniles (Ohara *et al*., 2005;

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Ozaki *et al*., 2013; Aoki *et al*., 2014). Similar to aquaculture production of other marine fish, hatchery production of *Seriola* species in the U.S. and elsewhere has been hindered by high larval mortality (Woolley *et al*., 2014) and a propensity for growth heterogeneity and deformities developed during larval and early juvenile stages that limit the production capacity and efficiency. Growth heterogeneity may be especially problematic for *Seriola*, as it has been estimated in *S. lalandi*, for example, that small fish constitute up to 42% of an offspring population, with many of those individuals exhibiting developmental issues leading to their demise (Moran, 2007).

Although other aspects of research (e.g., disease, nutrition, environmental factors, tank conditions) have supported the development of the yellowtail aquaculture industry, genetic improvement programs for *Seriola* culture are in the beginning stages (Ozaki *et al*., 2013). To date, genetic research aimed at improving *Seriola* aquaculture has included: construction of linkage maps at varying levels of coverage (including a map utilizing a hybrid cross between *S. quinqueradiata* and *S. lalandi*, Ohara *et al*., 2005), development of genetic markers (microsatellites and single nucleotide polymorphisms (SNPs)), identification of markers associated with candidate genes of interest, mapping of genes, estimation of heritability for commercially-relevant traits, and identification of quantitative trait loci (QTLs) associated with disease resistance (Ozaki *et al*., 2013; Whatmore *et al*., 2013; Aoki *et al*., 2014; Fuji *et al*., 2014). However, up until this point, bioinformatics data resulting from next generation sequencing has not been applied toward improving aquaculture for *Seriola*. The development of large-scale genomic resources has become increasingly accessible and affordable for non-model organisms. These types of genetic resources have been developed and used extensively in agriculture and livestock breeding for decades to improve product quality and quantity. Only more recently have these genomic approaches been applied to select aquaculture species (e.g. rainbow trout, Atlantic salmon, tilapia, catfish, flounder, Atlantic cod) (Terova *et al*., 2013; Dunham *et al*., 2014). Applying these genomic approaches to *Seriola* would greatly aid in the selection for economically important traits (Aoki *et al*., 2014) and improve the understanding of the biological, biochemical, and molecular networks involved in larval development, which could then be used to improve culturing techniques (Benzekri *et al*., 2014; Mazurais *et al*., 2011).

In this study, we describe the generation of whole larval transcriptomes for the California yellowtail (*S. dorsalis*) at three developmental stages: 2, 7, and 17 days post hatching (dph) using RNA-Seq on the Illumina HiSeq 2500 sequencing platform. Transcriptional profiles and differential gene expression were also investigated for slow- and fast-growing larvae over these same stages. We will present the findings of the transcriptomic investigations. Through this study, we hope to apply the gene expression results to generate a better understanding of the mechanisms and timing of larval development in *S. dorsalis*, and of the genes and processes involved in the observed growth heterogeneity that has limited *Seriola* aquaculture production.

Methods

Larval yellowtail were collected from spawning events using the wild-caught broodstock population held at Hubbs-SeaWorld Research Institute (HSWRI) (San Diego, CA). Yellowtail larvae were sampled opportunistically at 2, 7, and 17 dph with three replicates for each time point, and the replicates within each time point were sampled from separate spawning events. Depending on developmental stage, one to several larvae were placed on a microscope slide and euthanized with a lethal dose of MS-222. The larvae were quickly photographed, measured, and sorted by size into vials containing RNAlater® (Ambion). The vials of RNAlater® were kept on ice until being placed in -20 °C storage at the NMFS laboratory.

As a proxy for physical larval fitness (i.e., overall larval growth and robustness), the smallest and largest larvae, hereafter referred to as slow-growing (SG) and fast-growing (FG) larvae, were sampled within each spawning event at the three time points used for this experiment, with three replicates at each time point. For each replicate, 10 whole larvae

were pooled for the tissue homogenization. Pooling was used to: 1) minimize differences between individual larvae and focus on general differences in larval age and fitness groups and 2) provide adequate amounts of RNA for the analyses, which was problematic in single specimens at the earlier time points (i.e., 2 dph). Whole larvae were utilized at the three time points to best characterize organism-wide developmental changes, particularly as many organ systems were not developed at 2 dph.

RNA-Seq was conducted on the Illumina Hiseq 2500, and a total of 600 million reads were generated for the 18 samples (30 million reads/replicate). Raw read data were assembled using Trinity (Haas *et al*., 2013), and DESeq (Anders & Huber, 2010) was used to calculate the differential expression (DE) of the genes for every combination of timepoint and group (SG and FG fish). For the exploration of gene ontology (GO) enrichment, only transcripts with a fragments per kilobase of exon per million fragments mapped (FPKM) value of greater than four in at least one of the 18 samples was retained, resulting in a total of 54,858 high confidence transcripts. GO enrichment was performed on each subset of DE genes identified by DEseq for each comparison with a false discovery rate (FDR) corrected q-value cutoff of 0.05. GO enrichment was also performed on additional subsets of transcripts that had opposite transcriptional trends spanning the three time points between the SG and FG samples. The transcripts were required to have at least 15 normalized read counts across all 18 samples, and there had to be at least a twofold change between the averaged counts of biological replicates for days two and 17, which represented the start and end points of the sampling. GO terms were further analyzed for broad GO term categories using CateGOrizer and MGI_GO_slim2 categories (Hu *et al*., 2008), and pathway analysis was conducted using PathVisio 3 (Kutmon *et al*., 2015).

Lemon Tree analysis (Bonnet *et al*., 2015; Joshi *et al*., 2009) was performed to identify gene co-expression modules and assign regulators to those modules. Counts (FPKM) were normalized for library size, using RSEM (Li and Dewey, 2011). All 18 samples were used for clustering. Transcripts were filtered for having at least one sample with FPKM greater than four, resulting in 47,609 transcripts. Modules with significant interaction terms (FDR = 0.1) were selected for further analysis. Modules were sorted by magnitude of difference between groups in mean expression change over time. Modules with large expression increases over time in FG fish and large decrease over time in SG (and vice versa) were of particular interest in order to identify developmental gene differences or regulatory differences between these two groups. Module genes were pooled by pattern as described above. GO enrichment analysis (FDR = 0.05) was then performed within Cytoscape (v 3.2.1) (Smoot *et al*., 2001; Shannon *et al*., 2003) using the BiNGO (Maere *et al*. 2005) plugin, and zebrafish gene ontologies (http://zeogs.molgen.mpg.de/). Non-regulatory transcripts with ZFIN (Zebrafish Information Network) (Sprague *et al*., 2005) gene IDs were used as the reference gene set for enrichment analysis.

Results and discussion

For slow-growing (SG) fish between 2 and 7 dph and between 7 and 17 dph, 343 and 765 significantly enriched GO categories were identified, respectively. In fast-growing (FG) fish, 604 and 904 GO categories were identified as significantly enriched between 2 and 7 dph and between 7 and 17 dph. For SG and FG fish at both time period comparisons (2-7 dph and 7-17 dph), the GO categories of metabolism and catalytic activity contained the largest proportion of enriched terms (Fig. 1).

A large proportion of enriched terms fell under the transport category for all but the 2-7 dph SG fish, where it was significantly lower than other comparisons. Interestingly, the proportion of enriched terms in the cellular metabolism category was significantly higher in SG fish (both time periods) than in FG fish. Within the cellular metabolism category, nucleic acid metabolism was higher for 2-7 dph and 7-17 dph SG fish (5.1% and 4.6%, respectively) than for FG fish (1.8% and 1.9%). However, enriched GO terms under primary metabolism were higher between 7-17 dph in the FG fish but not in SG fish and not different between SG and FG fish in the 2-7 dph comparisons (Fig.1).

The normalized count data for the three biological replicates at each time point were averaged. These

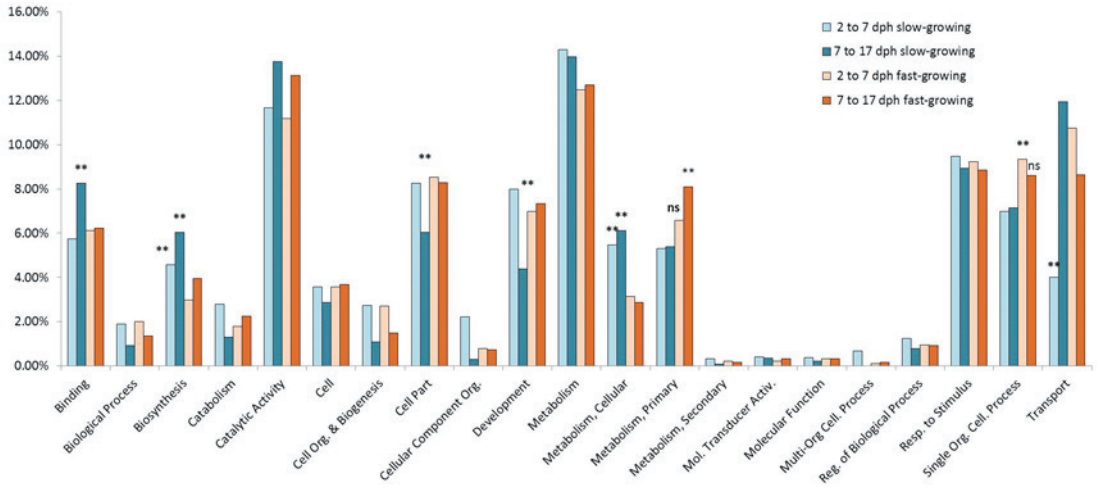

Fig. 1. Proportions of enriched DE GO terms assigned to GO categories between two time periods, 2 to 7 days post hatch (dph) and 7 to 17 dph, for SG and FG fish. Significant differences in GO categories between growth category or time period are represented by * ($p < 0.05$) and ** ($p < 0.01$), and ns (not significant) is used to clarify significance relationships when necessary.

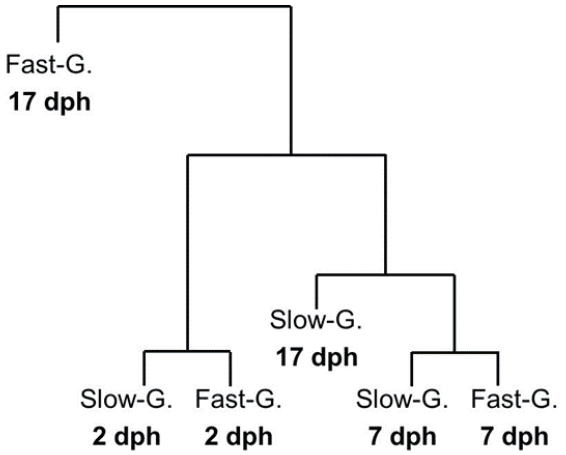

Fig. 2. A hierarchical clustering dendogram for all gene transcripts to visualize the overall similarity between time points of slow-growing (SG) and fast-growing (FG) fish.

averaged values were then used to generate a hierarchical clustering dendrogram using hclust (method = "average") (Murtagh and Legendre, 2014; Murtagh, 1985) to visualize the overall similarity between time points of SG and FG fish. Similar to the phenotypic characterizations we used to classify the SG and FG fish, we note the transcriptional profiles of 2 dph and 7 dph cluster together, whereas by 17 dph, the FG fish show a significantly different transcriptional profile than the SG fish. In fact, the SG fish at 17 dph are transcriptionally more similar to 7 dph and 2 dph FG and SG fish than to FG fish at 17 dph (Fig. 2).

In the module analysis, initial clustering produced 878 modules, and 63 of these had significant interaction terms (FDR = 0.1). Genes from these modules were further grouped into expression categories: 1) up-regulation in FG and down-regulation in SG fish (31 modules) and 2) up-regulation in SG and down-regulation in FG fish (12 modules). For modules showing preferential increases in FG fish, top GO terms (and associated top hierarchical enriched GO terms within the GO network) for module genes were regulation of metabolic process, regulation of biological process, biosynthetic process, ribosome biogenesis, nucleobase-nucleoside-nucleotide and nucleic acid metabolism, response to hypoxia, mesenchyme cell migration, cellular component assembly, small molecule metabolism and generation of precursor metabolites and energy, and transport. The percentage terms under the GO categories of translation, generation of precursor metabolism and energy, cellular nitrogen-compound metabolism, generation precursor metabolites and energy, biosynthetic process, and small molecule metabolism were significantly higher in FG fish than in SG fish (Fig. 3).

For the 12 modules exhibiting differential increases

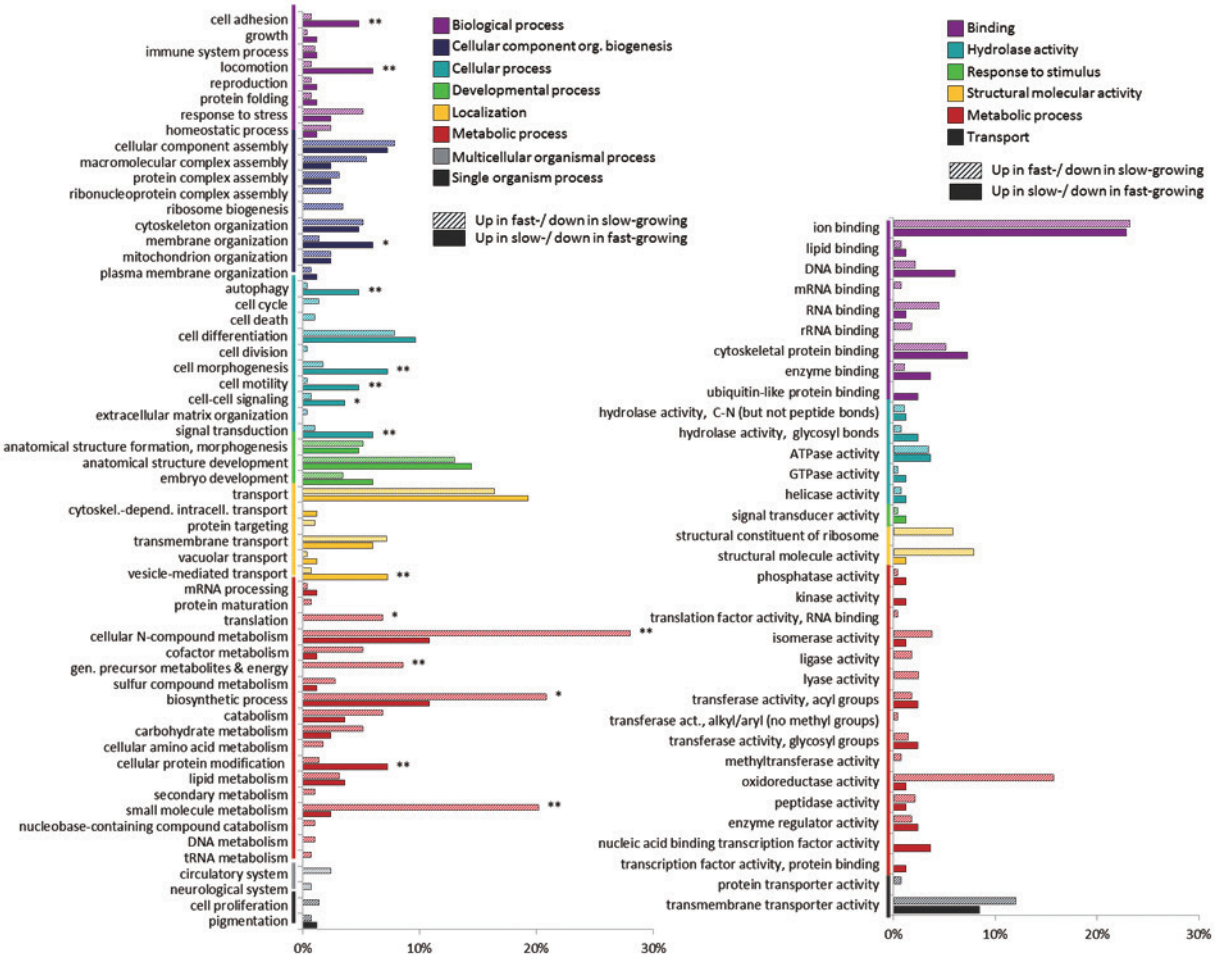

Fig. 3. From the significant modules, the proportions of terms identified in GO categories for genes in biological processes (A) and molecular function (B). Bar color reflects inclusion in the broader parent GO categories listed in the key provided for each graph. GO categories significantly higher for either the FG or SG larvae are denoted by *($P < 0.05$) and ** ($P < 0.01$).

in SG fish, enriched GO terms associated with the non-regulatory genes included response to nutrient levels (starvation), cellular response to stimulus, autophagy and Schwann cell development. Terms under GO categories associated with cell adhesion, locomotion, membrane organization, autophagy, cell motility, cell morphogenesis, vesicle-mediated transport, cell-protein modification, signal transduction, and cell-cell signaling were significantly higher in SG fish module genes (Fig. 3).

Pathway analysis of SG and FG larvae at the time points revealed two pathways with obvious trends in developmental progression: oxidative phosphorylation (Fig.4) and the electron transport chain. The overall trend for these pathways included higher gene expression for most genes in these pathways in SG fish at 2 dph and a complete reversal by 17 dph, with primarily higher gene expression in FG fish. Pathway analysis within growth groups indicate that SG fish show decreasing gene expression in these pathways as development continues, while FG show increasing gene expression over the same 15 day period.

It is not immediately obvious why 2 dph SG larvae exhibit up-regulation in oxidative phosphorylation and electron-transport chain pathways compared to FG larvae at the same time point. These larvae appear to be generating or attempting to generate higher levels of energy that do not translate into growth. Quickly the SG larvae lag behind the FG larvae, in size and in developmental stages; instead they up-regulate processes related to starvation responses and autophagy. One hypothesis is that one

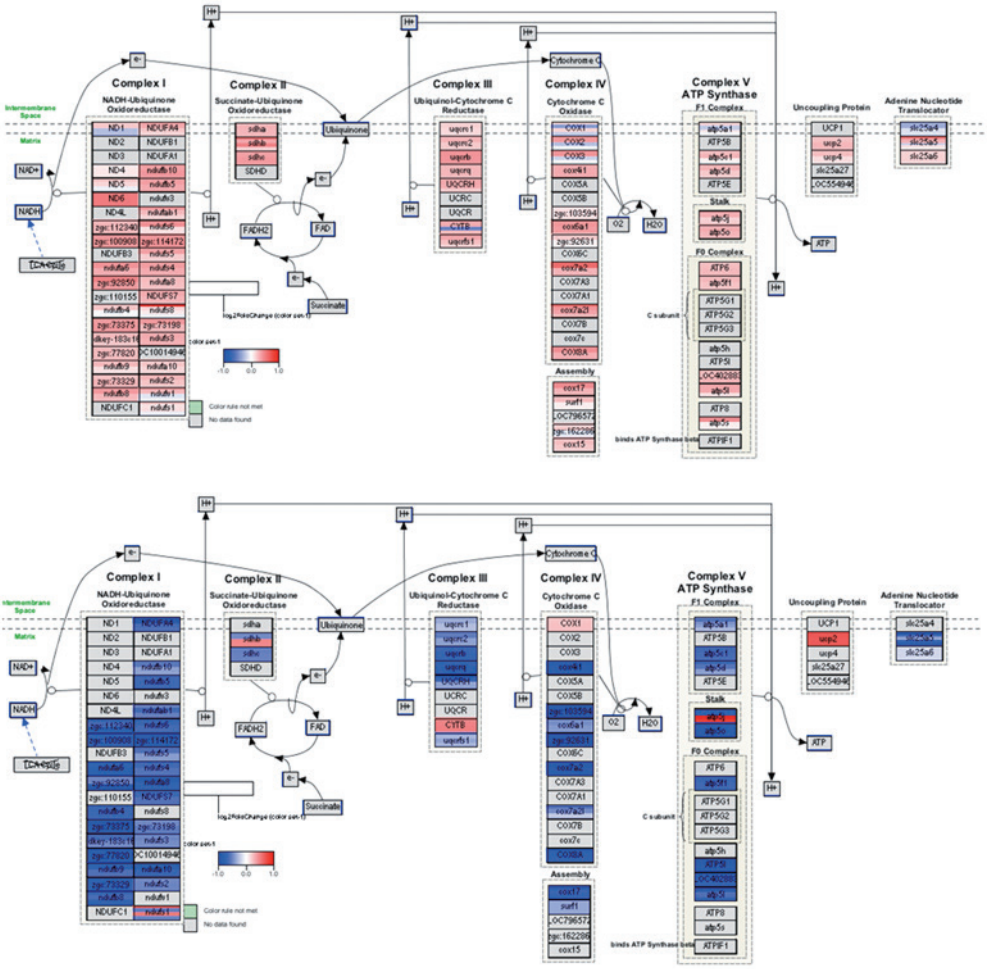

Fig. 4. Gene expression comparison (insert expression info) in the electron transport chain pathway between 2 dph (A) and 17 dph (B) FG and SG larvae. Gene boxes in pink/red indicate higher gene expression in SG larvae, and gene boxes in blue indicate higher gene expression in FG larvae.

or more of the ATP-generating pathways contain a mutation or some alteration that makes the pathway(s) less efficient, and instead of generating energy, these processes end up taking a higher metabolic toll on the SG larvae (Meyer and Manahan, 2010; Kocmarek *et al*., 2014). Alternatively, it has been documented that starvation can result in different metabolic adaptations in fish larvae (Salem *et al*., 2007), and it may be possible that the up-regulated gene expression patterns may result from inabilities to translate yolk-reserves into energy in the egg or yolk-sac larval stages. Next steps in this research will include exploring significantly associated regulatory genes, investigating the stages earlier than 2 dph, and examining mitochondrial function in the slow-growing larvae.

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(1) Kocmarek A., Ferguson M., and Danzmann R., 2014: Differential gene expression in small and large rainbow trout derived from two seasonal spawning groups. *BMC Genomics* **15**, 57.

In this study, the authors wanted to identify genes that showed similar expression patterns between large and small rainbow trout from different spawning seasons in two types of tissue: liver and white muscle. The goal in identifying these genes was to be able to develop growth-related markers for use in breeding programs. They hypothesized that 1) genes related to carbohydrate and lipid metabolism, energy production, insulin, and growth factors would be down-regulated in both tissue types in the small fish, 2) that genes involved in cytoskeletal structuring would be down-regulated in small fish, while myostatin will be up-regulated, and 3) that liver-specific lipid binding, cytoplasmic components, signaling, and transcription would be up-regulated in small fish. They observed that genes related to immune function were up regulated in large fish; suggesting that enhanced growth is associated with enhanced immune function. They also found that genes related to transcription, translation, and protein production were up regulated in small fish (from Sept.) in white muscle, which supports patterns previously detected in liver tissue. This indicates that protein production in small fish may not be translating effectively into finished proteins. This study was able to identify patterns of differential gene expression in small and large rainbow trout; this will enable future studies to delve deeper into the genes related to differences in growth.

(2) Meyer E. and Manahan D., 2010: Gene expression profiling of genetically determined growth variation in bivalve larvae (*Crassostrea gigas*). *J. Exp. Biol.* **213**, 749-758.

The authors compared gene expression patterns in larvae of the Pacific oyster (*Crassostrea gigas*) that exhibited slow- and fast-growth (these larvae were produced from experimental crosses). Based on a previous transcriptome-wide analysis, a set of 181 candidate genes for growth heterogeneity were analyzed with the goal of understanding the biological processes underlying the differential growth rates. Of the genes identified by GenBank, ribosomal proteins were the most abundant, comprising 50% of the total genes with 17 different ribosomal protein genes. The genes included nine components of the large ribosomal subunit, and eight components of the small ribosomal subunit. Some of these genes were up-regulated in the fast-growing larvae (n = 6), while others were up-regulated in the slow-growing larvae (n = 11). Since ribosome biogenesis is a significant metabolic cost in cell proliferation, any changes in this pathway would likely have a large effect on the

overall energy metabolism. The authors hypothesize that in the slow-growing larvae there may be a high metabolic cost to synthesizing and degrading excess ribosomal protein copies resulting from the higher expression of those genes.

(3) Moran D., 2007: Size heterogeneity, growth potential and aggression in juvenile yellowtail kingfish (*Seriola lalandi Valenciennes*). *Aquac. Res.* **38**, 1254-1264.

In this study, the authors are describing the occurrence of size heterogeneity and aggressive behaviors in cultures of *Seriola lalandi*; this was done to examine the effectiveness of size-grading in reducing aggression and mortality, and increasing growth rates. To do this, graded and ungraded juveniles were compared for various measures of aggression and growth, and a RNA/DNA ratio was used as a measure of growth rate. The authors found that size heterogeneity became more pronounced at 12 days post hatch (dph) when *Artemia* are offered as a food source. While the large and aggressive juveniles only accounted for 8% of the population, the small grade juveniles that received the aggression accounted for 42% of the population. In the ungraded treatment, this aggression was associated with mortality for most small fish. However, even without aggression, the small-grade juveniles did not gain weight or increase their RNA/DNA ratio after 12 days. The authors believe that these small fish appear to be on a degenerative developmental strategy without any influence of the larger aggressive fish.

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

The authors of this study used microarrays to identify genes and pathways involved in the starvation response and protein turnover in rainbow trout, and to identify metabolic adaptations that occur in the liver during these starvation periods. This study was of interest because, as the authors point out, examining changes in metabolism during starvation is an effective way to identify relationships between metabolic pathways and body processes. The experiments showed down-regulated expression of genes involved in protein biosynthesis in the starved fish, but no significant changes in protein catabolic pathways, and a slight increase in 20S proteasome activity. Responses in the liver to starvation included an overall decline in gene expression associated with decreasing tissue metabolism, a reduction in protein synthetic capacity, an impairment of ATP-biosynthesis, and lower expression in hepatic lipid and fatty acid transport.