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M. J. Darias[1], J. L. Zambonino-Infante[1], K. Hugot[2], C. L. Cahu[1] and D. Mazurais[1, *]

[1] Ifremer, Nutrition Aquaculture and Genomics Research Unit, UMR 1067, Ifremer, Technopole Brest-Iroise, BP 70, 29280 Plouzané, France
[2] INRA, DGA, UMR314, Laboratoire de Radiobiologie et d'Etude du Génome, CRB GADIE, INRA de Jouy-en-Josas, F-78352 Jouy en Josas, France

*: Corresponding author : D. Mazurais, tel : 33-298224487, fax : 33-298224366, email address : David.Mazurais@ifremer.fr

Abstract:

During the larval period, marine teleosts undergo very fast growth and dramatic changes in morphology, metabolism, and behavior to accomplish their metamorphosis into juvenile fish. Regulation of gene expression is widely thought to be a key mechanism underlying the management of the biological processes required for harmonious development over this phase of life. To provide an overall analysis of gene expression in the whole body during sea bass larval development, we monitored the expression of 6,626 distinct genes at 10 different points in time between 7 and 43 days post-hatching (dph) by using heterologous hybridization of a rainbow trout cDNA microarray. The differentially expressed genes (n = 485) could be grouped into two categories: genes that were generally up-expressed early, between 7 and 23 dph, and genes up-expressed between 25 and 43 dph. Interestingly, among the genes regulated during the larval period, those related to organogenesis, energy pathways, biosynthesis, and digestion were over-represented compared with total set of analyzed genes. We discuss the quantitative regulation of whole-body contents of these specific transcripts with regard to the ontogenesis and maturation of essential functions that take place over larval development. Our study is the first utilization of a transcriptomic approach in sea bass and reveals dynamic changes in gene expression patterns in relation to marine finfish larval development.

Keywords: Fish larvae - Development - Microarray - Gene expression - Sea bass

1. Introduction

European sea bass is an economically important fish species whose aquacultural production has more than doubled in Europe during the last decade. Part of this increase in production can be attributed to scientific progress in breeding and husbandry, and to the development of new methodologies aiming to optimize rearing conditions. However, larval rearing methods are still unreliable, with highly variable malformation and survival rates. There is clearly therefore a need to improve our understanding of the cellular processes underlying marine larval development.

Sea bass larvae undergo dramatic changes in body shape, morphology, metabolism, swimming abilities and behavior until their metamorphosis into juveniles is complete, at around 40 days post hatching (dph). Largely, post embryonic development will transform an organism that initially has only a rudimentary or non functional liver, pancreas, gallbladder and spleen, undifferentiated skeleton, incompletely developed nervous system and no gills, into a juvenile with a mature digestive tract and developed nervous system, calcified skeleton, final fins, finrays, a thick skin and functional gills (Falk-Petersen, 2005). Studies performed on marine fish have particularly demonstrated the importance of enzymatic regulation in the digestive tract (Zambonino-Infante and Cahu, 2001) and variation in energetic metabolism (Wieser, 1995) during larval development. However, more exhaustive molecular approaches are needed to identify the genes involved in regulation of the key biological changes occurring during marine fish larval development. There is indeed a consensus that regulation of gene expression is a fundamental mechanism underlying the complex processes of cell differentiation and morphogenesis during ontogenesis of an organism.

Recently, microarrays have dramatically accelerated many types of investigations in marine organisms, been especially used for comparative genomic hybridization (Fish; Moriya et al., 2004 and 2007) and for monitoring the expression levels of thousands of genes simultaneously in order to study the effects of certain treatments (Algae; Jamers et al., 2006), diseases (Crustaceans; Wang et al., 2006) and transgenesis manipulation (Mori et al., 2007) on transcriptome.

Over the last two decades, studies based on whole-body or tissue-specific microarray approaches have investigated gene expression profiles during embryogenesis in several species (*Drosophila*, White et al., 1999; *Xenopus*, Baldessari et al., 2005; mouse, Tanaka et al., 2000) including fish (zebrafish, Ton et al., 2002; sea bream, Sarropoulou et al., 2005). However, until now, no study has focused on characteristics of a marine fish transcriptome during larval development and metamorphosis. Newly hatched marine fish constitute an interesting and original model since they probably are the fastest growing of all vertebrates. A transcriptomic approach based on the large scale investigation of gene expression through cDNA microarray hybridization is particularly appropriate for the study of ontogenesis in such complex organisms, since the development of their different organs and functions are closely related in early larval development.

As no DNA microarray is presently available for sea bass, we adopted a heterologous approach for our transcriptomic analysis of larval development. This strategy, using microarrays developed on other species, has already been successfully employed with other fishes (Cohen et al., 2007; Kassahn et al., 2007; Renn et al., 2004; Von Schalburg et al., 2005). Interestingly, in a recent paper, Cohen et al., (2007) have experimentally and theoretically evaluated the relative efficiency of cross-species hybridizations across bony fishes. Their results showed that fish cross-species cDNA microarray hybridizations at family or suborder levels are quite beneficial tools for investigating gene expression patterns. The data they obtained from cross-species hybridizations among fish of different taxonomic families (cDNAs of three-spine stickle backs on a European flounder microarray) revealed also 65% intra-superorder (Acanthopterygii) efficiency as compared to monospecific hybridization. In the same way, the purpose of previous experiments performed within the frame of a French national program (genofish) was to evaluate cross-species (rainbow trout-sea bass) cDNA microarray hybridizations at subdivision order (Euteleostei). These experiments revealed that hybridization of generic trout cDNA microarray with cDNA of sea bass larvae could be considered with regard to the percentage of hybridization signal obtained (25% of total spotted cDNA displayed hybridization signal; data not shown).

Accordingly, we used in the present study cDNA microarray developed from rainbow trout pooled-tissue libraries, containing 9023 cDNA clones, to which we hybridized 30 samples obtained at 10 stages during sea bass larval development. In order to minimize the risk of non-specific hybridization signals, heterologous hybridization was carried out following the same protocol as that usually used for a homologous approach. We analyzed the genes that were differentially expressed through larval development and treated the expression data to establish which biological processes were enriched with respect to regulated genes. This study gives a panoramic view of the complex changes in gene

expression occurring over larval development, and provides new insights into the dynamic regulation of biological processes related to organogenesis, energetic pathways, biosynthesis and digestive functions at specific ontogenic stages in a commercially important species.

2. Materials and Methods

2.1. Animals

Three-day-old post hatching (3 dph) European sea bass (Dicentrarchus labrax) larvae were obtained from the Ecloserie Marine de Gravelines (France). These larvae were acclimated in one 250 L-cylindroconical fiber glass tank at an initial density of 80 larvae/L (20 000 larvae/tank). Tanks were supplied with through-flowing sea water, which had been previously filtered through a sand filter, then passed successively through a tungsten heater and a degassing column packed with plastic rings. Throughout the experiment, salinity was 35 ‰, temperature was 20°C and the oxygen level was maintained above 6 mg/L by setting the water replacement in the tank at up to 30% per hour (flow rate: 0.18 L/min). All procedures concerning the animals and their handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985). Throughout the experimental period, larvae were continuously fed with a patented diet (WO 0064273) in large excess for 24 hours per day using automatic belt feeders.

2.2. Larval sampling and RNA collection

Three pools containing 100-150mg of larvae (the number of larvae in the samples depended on their developmental stage) were randomly collected at days 7, 9, 13, 17, 23, 25, 27, 31, 35 and 43 dph and immediately frozen in liquid nitrogen. Total RNA for each pool was extracted using the TriZol protocol (Invitrogen), and the quantity and quality of extracted RNA analyzed using a spectrophotometer (ND-1000, Nanodrop) and Bioanalyser (Agilent) respectively.

2.3. cDNA microarrays production

Gene expression analyses were performed using Nylon DNA microarrays obtained from INRA-CRB GADIE (Jouy-en-Josas, France) resource center. These trout microarrays contained 9216 DNA spots encompassing 9023 trout cDNA clones and a set of 193 controls. Trout cDNA clones are originated from 2 pooled-tissues libraries (Govoroun et al., 2006). Negative controls consisted of 128 spots of a Photinus pyralis modified luciferase clone which is devoid of similarity with fish DNA sequences, 1 spot of a Arabidopsis thaliana GAG1At cDNA clone and 64 water spots. This generic array is deposited in Gene Expression Omnibus (GEO) database (Platform# GPL3650) (http://www.ncbi.nlm.nih.gov/projects/geo/). Trout cDNA clones were spotted onto nylon membranes after PCR amplification as previously described (Nguyen et al., 1995). All these clones were PCR amplified using primers designed on the plasmid sequences flanking the cDNA inserts (M13RP1 5'-GTGGAATTGTGAGCGGATAAC and M13RP2 5'-GCAAGGCGATTAAGTTGGG). 35 cycles of PCR amplifications were carried out in 100 µl of 1× buffer containing 1.5 mM MgCl2, 100 µM dNTP, 100µM of each primer, and 5 Units of Taq polymerase (M1865,Promega, Madison, WI). Quality of the amplification products was systematically checked on 1% agarose gels. Unpurified PCR products were evaporated, resuspended in 20µl of water, then spotted onto nylon membranes (Hybond-N+; Amersham Biosciences, Saclay, France) using a Biorobotics MicroGrid-II arrayer (Genomics Solution, Cambridge, U.K.) equipped with a 64-pins Biorobotics printhead and 64 Biorobotics 100µm solid pins. The spotted DNA was denaturated in 150mM NaOH, 1.5M NaCl. Neutralisation step was performed in 1M Tris HCl (pH 7.5), 1.5M NaCl. After rinsing micromembranes in 2X SSC, the DNA was fixed by successive heat (80°C during 2 hours) and UV (120000µJ) treatments.
All DNA microarrays used in this study were made at the same time and under the same conditions. The microarray has been successfully used to reveal gene expression patterns in trout testis and ovary (Baron et al, 2005; Bobe et al., 2006 ; Bonnet et al., 2007).

2.4. Identity of mircroarray cDNA clones

Sequences originating from trout libraries were used to generate publicly available contigs (http://www.sigenae.org). BlastX was performed by comparison of contigs sequences against the Swiss-Prot database (http://www.expasy.org/sprot/). This was performed automatically for each EST spotted onto the membrane and used to annotate the 9023 clones of the microarray.

2.5. Hybridization to cDNA microarrays

To determine the relative amount of DNA accessible for hybridization on each spot, 'vector-hybridization' with an oligonucleotide (5'-CACTATAGGGAATTTGGCC-3') was first performed, as previously described by Mazurais et al. (2005). After stripping (3 hours 68°C, 0.1× SSC, 0.2% SDS), membranes were prehybridized for 6 hours at 65°C in hybridization solution (5× Denhardt's, 5× SSC, 0.5% SDS, 100µg/ml denatured calf bovine thymus) and then hybridized 48 hours in hybridization buffer with radiolabeled complex probes. The complex probes were labeled by reverse transcription using 5 µg RNA in the presence of 50 µCi [alpha-33P] dCTP, 5 µM dCTP, 0.8 mM each dATP, dTTP, dGTP and 200 units M-MLV SuperScript RNase H-reverse transcriptase (GIBCO BRL) in 30 µL final volume. RNA was degraded by treatment at 68°C for 30 minutes with 1 µl 10% SDS, 1 µl 0.5 M EDTA and 3 µl 3 M NaOH, then equilibrated at room temperature for 15 min. Neutralization was achieved by adding 10 µl 1 M Tris- HCl plus 3 µl 2N HCl.
After hybridization, membranes were washed (3 X 1 hour at 68°C in 0.1× SSC, 0.2% SDS). Phosphor-imaging plates were exposed to the microarrays for 65 hours and scanned using a FUJI BAS 5000. Signal intensities were quantified using AGScan software derived from BZScan2 (http://tagc.univ-mrs.fr/ComputationalBiology/bzscan/) developed at the TAGC laboratory (Marseille, France) (Cathelin et al., 2007).

2.6. Microarray signal processing

The complete raw dataset of the 9023 trout cDNA clones is available on marine biotechnology web site (complementary folder 1).
Filtering: In order to ensure hybridization accuracy, the quality of the signal obtained for each spot was assessed using control measures in the AGScan software. The software provides a qualitative flag for each spot which evaluates if the feature's shape is spot-like or not, and a quantitative quality metric on a 0 to 1 scale. This is used to evaluate the quality of the measured signal, compared to an ideal radioactive spot. Quality spot determination is fully described in technical notes (http://tagc.univ-mrs.fr/bioinformatics/bzscan/bzscan-outputs/node15.html). Such treatment allowed us to flag weak or doubtful signals within samples and therefore avoid excessive noise generated by a large gene set. For expression pattern analysis of the genes regulated through larval development, we examined only the 1626 clones that displayed three high quality spots for at least one stage.
Corrections and normalization: As no variability was observed between the different membranes following vector-hybridization, we did not perform any correction of complex probe hybridization signals with the oligonucleotide values. To minimize variation arising from technical differences in RNA quality, probe labeling, and hybridization conditions between experiments, transformation and normalization were performed by Log and standardization, respectively (as described by Riva et al., 2005). Firstly, a logarithmic transformation for each signal intensities was performed in all filtered sets to obtain Log (hybridization values). Considering that the majority of gene expression should not change in function of the different experimental conditions and that the overall intensity change of up- and down-regulated genes should be similar, the total quantity of cDNA (sum of Log hybridation values) and the variance of Log (hybridization values) within each set should be the same across the different samples. For that reason, a median normalization has been performed by subtracting the median of Log (hybridization values) in a set (sample) from each Log (hybridization values) for that set (to obtain Log normalized hybridation values). Correction for differences in the variance across the range of gene expression levels were next done by dividing each Log (normalized hybridation values) by the standard deviation of the Log (normalized hybridation values) for each set. Figure 1 represents scatter plots between replicates for both the raw data and the normalized data. Normalized data concerning the 1626 analysed cDNA clones are available on the marine biotechnology web site (complementary folder 2).
For all genes analyzed, ontologies (biological processes) were obtained using EASE software version 2.0 tools (open source available at http://david.niaid.nih.gov/david/ease.htm).

2.7. Microarray data analysis

Expression patterns of genes corresponding to filtered, normalized and corrected hybridization values were first assessed using unsupervised hierarchical clustering with the program Gene Cluster developed by Michael Eisen (http://rana.stanford.edu/software). The mean of the three values for each stage was used for clustering treatment. Tree View software, also available at the Stanford site, was used to generate visual representations of the classification.
Genes differentially expressed during larvae period were next determined statistically (False Discovery Rates < 3%) by variance analysis using Genanova software (developed in CNRS UPRESA 8087, France).
Based on ontology annotation using EASE version 2.0 tools (Hosack et al., 2003), the functional categories of the regulated genes were used to determine which biological processes were enriched (with p value <0.05). Over-represented gene categories were determined using EASE score which is a conservative adjustment to the Fisher exact probability that weights significance in favor of themes supported by more genes. The theoretical basis of the EASE score lying in the concept of jackknifing a probability is detailed by Hosack et al., 2003.
Expression pattern of genes involved in selected biological processes were then visualized using supervised clustering applied on samples classified according to developmental stage.

2.8. Real time PCR analysis

Expressions of trypsin, collagen 1α2 and Amylase 2 were investigated by Real-time PCR at days 9, 17, 27, 35, 43 dph. Amplifications were performed from RNA samples used for microarray analysis. Reverse transcription and real time PCR were monitored as previously described (Villeneuve et al., 2006) using the MyIQ single color real time PCR detection system (Biorad). Reverse transcription products were diluted to 1/300, and 5 µl were used for each real-time PCR reaction. Triplicates were run for each RT product. Real-time PCR was performed using SYBR Green supermix (Biorad) according to the manufacturer's instructions. The level of EF1α RNAs was monitored using the same sample set to allow normalization. Primers (Amylase 2: sense- GATCACCAGATGCAACAACG, antisense- CTGAACCAGCTTCCACATGA ; Trypsin: sense- CTCCCTGGTCAACGAGAACT; antisense- ACCCTGATGTTGTGCTCTCC; collagen 1α2: sense- TCGCCCAGAAGAACTGGTACAGAA, antisense-CGTTGTAGGTAAACTCAGTACCACCG; EF1: sense- GCTTCGAGGAAATCACCAAG, antisense- CAACCTTCCATCCCTTGAAC) were designed from sequences available in Genbank database (Amylase 2: AJ310653, Trypsin: AJ006882, collagen 1α2: CX660451, EF1: AJ866727). Real time PCR data were treated using ΔΔCT method available in IQ5 software (Biorad).

3. Results and Discussion

3.1. Total expression data

In this study, we present the first large scale DNA microarray gene expression profile made on sea bass. Transcriptomic analysis was conducted throughout sea bass larval development using a trout microarray containing 9023 cDNA clones from pooled-tissue libraries. Such microarrays have already been successfully used with a homologous approach (Baron et al, 2005; Bobe et al., 2006 ; Bonnet et al., 2007). With 93% of clones annotated, this microarray represents one of the most reliable datasets currently available for a heterologous study in teleost species. As mentioned in the 'Materials and Methods', the quality of the spots obtained after hybridization was closely monitored using the AGScan software quality control measures, which allowed us to cut down the number of genes in the set by eliminating those with weak or doubtful signals. Considering that regulated genes could display a weak expression level through most of larval development but be highly expressed at just one stage, we filtered out clones that did not display three high quality spots (one per sample) at least one stage. The final dataset contained 1626 expression patterns. These 1626 clones correspond to 1278 distinct genes (21% of the 6626 distinct genes represented on the array) that are conserved between the two species and display a sufficient expression level in sea bass larvae to be detected by hybridization of whole-larvae RNA samples. Gene ontology annotation showed that these 1278 genes are involved in 914 distinct biological processes (listed in complementary folder 3). The variety of these processes can be explained by the nature of the samples analyzed (RNA extracted from whole larvae), as well as by the nature of the array (cDNA from pooled and multi-stage tissue libraries). As expected, some of

these 914 biological processes are related to cell growth and maintenance, morphogenesis, biosynthesis and development. **Table 1** summarizes the 15 most abundant gene ontology categories (biological process) associated with these genes. This overview suggests that our protocol for hybridization of sea bass complex target to spots of trout cDNA is a functional heterologous technique. However, even if the cDNA probes employed here are likely to be less sensitive to sequence mismatches than oligonucleotide probes, we can not exclude the possibility that gene dependent sequence divergence between the two species influenced hybridization intensity on some of the spotted clones (Buckley, 2007). In all, these observations strongly suggest that more than 1626 cDNA clones would have been detected using an homologous approach.
This heterologous hybridization was carried out using the same protocol usually used for homologous approaches, in order to minimize the risk of non-specific hybridization signals. The accuracy of the methodology applied here is confirmed by the homogeneity of the expression profiles between redundant genes involved in distinct biological processes [*Trypsin I* (*TRY1*), *Glyceraldehyde-3-Phosphate dehydrogenase* (*G3P*), *Hemoglobin alpha subunit* (*HBAE*)] (**figure 2a**), as well as by the consistency of the hybridization data with the expression profiles of the genes (*collagen1 alpha 2*, *trypsin*, *amylase2*) additionally analyzed by real time PCR (**figure 3**).
The main factor limiting a reliable interpretation of expression data is the nature of complex targets prepared from whole-body RNA extracts. In order to correctly interpret the results, it is important to keep in mind that the expression profiles of individual transcripts reflect the expression level of the corresponding genes in a mixture of cell types and tissues. Thus the variation in expression observed in our experiments could reflect changes in proportions of different tissues during development.

4. Global Analyses

Global hierarchical clustering was first applied on the 1626 high quality hybridization signals (**figure 2b**). This clustering was used to classify samples according to their overall gene expression profiles on the horizontal axis, and to group genes on the basis of similarity of their expression profile in all samples on the vertical axis. Hierarchical clustering allowed us to assess the repeatability of the method (**figure 2a**) and to identify large gene clusters that were over- or under-expressed in the different samples. It appeared that numerous genes were up-expressed during final steps of development (orange bar) compared with other clusters encompassing genes up-expressed early during development (blue bar). These results confirmed that regulation of gene expression is a key mechanism underlying morphogenesis and maturation of physiological functions occurring during ontogenesis of sea bass larvae. Based on these expression patterns, samples were grouped into two large clusters, the first group encompassing most larval samples taken from 7 to 23 dph, the second containing those sampled from 25 to 43 dph. The period ranging from 17 to 31 dph, during which the larval transcriptome underwent large changes, could correspond to the maturation of physiological functions and associated changes in the energetic metabolism already described in some previous studies (Zambonino-Infante & Cahu, 2001; Parra & Yufera, 2001).
Usually, this type of overall classification cannot detect differential gene expression between samples properly, due to the excessive noise generated by unregulated genes. Statistical analyses were thus performed to identify genes displaying significant differential expression through larval development.

4.1. Significant variation in gene expression during larval development

Variance analysis identified 595 clones, corresponding to 485 distinct genes (listed complementary folder 4), which were differentially expressed (FDR<3%) during the larval development (38% of the 1278 studied genes). Their corresponding gene ontology indicated that these genes are involved in 664 biological functions. EASE software was used to determine which biological processes associated to the 485 genes were over represented ($p<0.05$) when compared to those obtained from the 1278 analysed genes. We then focused our attention on four main biological processes (organogenesis, the energy pathway, biosynthesis and digestion) listed in **table 2**. Associated gene expression patterns were studied using clustering analysis (**figure 4**).

4.2. Organogenesis

The 39 genes involved in the organogenesis process are basically concerned with the development of bone, muscle and nervous system development.

4.3. Bone development

Four genes related to bone development showed higher expression from 31 dph onwards. *Collagen alpha 1(I)* (*COL1A1)* and *2(I)* (*COL1A2*) *chain precursors* form the fibrils of tendons, ligaments and bones. In bones, the fibrils are mineralised with calcium hydroxyapatite (Weiner & Traub, 1986). The *connective tissue growth factor precursor* (*CTGF*) promotes proliferation and differentiation of chondrocytes (Erwin et al., 2006). Finally, *periostin* (*POSTN*) may play a role in the recruitment and attachment of osteoblast precursors (Horiuchi et al., 1999). This finding agrees with results on sea bass skeletal development (Darias et al., in preparation) in which the first signs of vertebral column ossification/mineralization occurred around 30 dph.

4.4. Neural development

Genes involved in ontogenesis of the visual and nervous systems [Beta crystallin A4 (CRYBA4), AP-2 alpha (TFAP2A), Platelet-activating factor acetylhydrolase IB alpha subunit (PAFAH1B1), SOX-3 (SOX3), Protocadherin alpha C2 precursor (PCDHAC2) and Tubulin beta-4 chain (TUBB4)] were generally up-expressed during the early stage of larval development (7-13 dph). Expression of these genes shows that nervous and visual systems are still developing during this part of the larval period. The drop in expression detected afterwards does not necessary mean that the process of nervous system formation declines, but could arise from the decrease in neuronal/whole-body tissue mass ratio occurring throughout larval development. As a matter of fact, we also observed genes related to nervous system development, that were up-expressed at the end of the larval period [Ectoderm-neural cortex 1 protein (ENC1), Glucose-6-phosphate isomerase (GPI), Transcription factor SOX-11(SOX11)]. This data pattern of neural development during sea bass larval development agrees with results of several studies on zebrafish, describing post-embryonic neural proliferation in the forebrain (Wullimann et al., 1999) and the development of peripheral sensory structures (Sapède et al., 2002). These developments could be related to the sophistication of sense organs, formation of muscular tissues and to the acceleration of movement reactions as the larval period progresses (Gibb et al., 2006).

4.5. Muscle development

A set of genes related to different aspects of muscle contraction was generally increasingly expressed during larval development, showing higher expression from 31-43 dph: myosins involved in muscle contraction [*Myosin heavy chain alpha and beta isoforms* (*MYH6 and MYH7*)*, Myosin light polypeptide 6* (*MYL6*)*, Myosin-binding protein C* (*MYBPC2*)]; troponins that are the central regulatory proteins of striated muscle contraction [*Troponin C* (*TNNC*)*, I* (*TNNI*) *and T* (*TNNT*)*, Tropomyosin 1 and 4 alpha chain* (*TPM4 and TPM1*)], *Calsequestrin* (*CASQ2*) involved in the regulation of muscle contraction and *alpha actin* (*ACTA1*) which is the major constituent of the contractile apparatus. A gene implicated in muscle relaxation was also found (*Parvalbumin alpha: PVALB*) and displayed a similar pattern of transcript expression. This gene expression pattern reflects the progressive development of muscular tissues during larval ontogenesis (Mascarello et al., 1995; Galloway et al., 1999), necessary for swimming activity. Furthermore, the increase in muscular activity was correlated with the overall change in expression of genes related to energetic metabolism (described below) that we observed during sea bass larval development.

4.6. Energy pathways

The energy pathway cluster was represented by 26 genes specifically involved in the tricarboxylic acid (TCA) cycle, neoglucogenesis/glycolysis and ATP synthesis. These genes fell into two main groups according to their expression patterns. Genes involved in the TCA cycle [*Aconitate hydratase* (*ACO2*), *Succinate dehydrogenase* (*SDHB*), *Succinyl-CoA ligase* (*SUCLG1*), *Citrate synthase* (*CS*)*, Malate dehydrogenase* (*MDH2*)*, Dihydrolipoyllysine-residue succinyltransferase* (*DLST*)*, Dihydrolipoyl dehydrogenase* (*DLD*)*, Acyl-CoA dehydrogenase* (*ACADM*)], ATP synthesis [*ATP synthases* β*,* γ *and subunit C* (*ATP5B*, *ATP5C1* and *ATP5G3*)] and neoglucogenesis [*Phosphoenolpyruvate*

carboxykinases 1 and 2 (*PCK1* and *PCK2*)] were generally up-expressed during the early stage (7-13 dph) of sea bass larval development. This finding reflects the larval strategy for obtaining the energy necessary for metabolism of maintenance and development. It is well established that fish larvae mainly use dietary lipids and proteins as their energetic source, which are provided by both endogenous and exogenous nutrition until yolk-sac resorption around 15 dph (Conceiçao et al., 1998; Ronnestadt et al., 1998). Our results suggest that assimilated amino acids are metabolized by the TCA cycle and related ATP-synthases to produce an energy supply, and also may contribute to glucose synthesis through phosphoenolpyruvate production from oxaloacetate. This aerobic energy production path, associated with active tissues (liver, gills, intestine), was progressively masked by the development of white muscular tissue (Segner et al., 1994) in which the anaerobic energy necessary for swimming activity is mainly supplied by the glycolysis pathway. In fact, we found that genes encoding most of the enzymes involved in glycolysis were increasingly expressed throughout larval growth [*Fructose-bisphosphate aldolase A* (*ALDOA*), *Glyceraldehyde 3-phosphate deshydrogenase (GAPDH)*, *Pyruvate kinase* (*PKM*), *α* and *β-enolases* (*ENO1* and *ENO3*), *Phosphoglycerate mutase 1 and 2* (*PGAM1* and *PGAM2*), *6-phosphofructokinase muscle type* (*PFKM*), *Triosephosphate isomerase* (*TPI1*), *L-lactate dehydrogenase A chain* (*LDHA*), *Glucose-6-phosphate isomerase* (*GPI*)] (**figure 5**). Accordingly, a gene encoding *creatine kinase* (*TCK1*) was also associated with this glycolysis-related gene expression pattern. This enzyme is involved in the regulation of ATP budget in muscular cells (Meyer et al., 1984). These results agree with previous data indicating that energy metabolism in early fish larvae is almost entirely aerobic and that the anaerobic capacity of the fast muscle fibres, initially low after hatching, increases during the transition from larva to juvenile (Wieser, 1995).

4.7. Biosynthesis

The biosynthesis cluster was represented by 67 genes that could be classified into two main groups. The first and largest group was composed of genes that were generally down-expressed through larval development, and were mainly messengers related to protein biosynthesis. The second group included the up-expressed genes, basically represented by transcripts involved in cholesterol and NTP biosynthesis.

4.8. Protein biosynthesis

The majority of genes included in the protein biosynthesis group were involved in translation process. A set of 20 ribosomal proteins (*RPL* and *RPS*), initiation and elongation factors [*Eukaryotic translation initiation factor 3 subunit 3* (*EIF3S3*), *Eukaryotic translation initiation factor 5* (*EIF5*), *Eukaryotic initiation factor 4A-I* (*EIF4A1*), *Eukaryotic translation initiation factor 3 subunit 4* (*EIF3S4*), *Elongation factor 1-beta* (*EEF1B2*)] were observed, displaying an expression pattern characterized by a consistently higher level of expression between 7-13 dph than afterwards. The decline in gene expression observed from 17 dph could be attributed to the progressive increase in muscular mass, resulting in a proportionally lower representation of the tissues actively involved in protein synthesis (i.e., liver and intestine). However, it is important to note that high protein synthesis could be also related to the high growth rate observed in the early stages of fish larval development (Person-Le Ruyet et al., 1991; Parra & Yufera, 2001) and to the priority given to proteins as the main energetic source at this time (Conceição et al., 1998).

4.9. Cholesterol and NTP biosynthesis

Several genes involved in cholesterol metabolism [3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), C-4 methylsterol oxidase (SC4MOL), Farnesyl pyrophosphate synthetase (FDPS)] as well as in NTP synthesis [Nucleoside diphosphate kinases A, B and 3 (NME1,2,3)] showed their highest expression from 31 dph onwards. Cholesterol plays a central role in many biochemical processes, being especially important for the metabolism of fat soluble vitamins, including vitamins A, D, E and K. It is the major precursor for the synthesis of vitamin D and various steroid hormones (including cortisol and aldosterone in the adrenal glands, and the sex hormones: testosterone, progesterone, the estrogens, and their derivatives). Thus, up-expression of genes involved in cholesterol metabolism that we observed through larval development can be related to variation in endocrine hormone concentrations described during fish ontogenesis (de Jesus and Hirano, 1992; Deane and Woo, 2003; Szisch et al., 2005). NTPs are involved in many metabolic processes such as nucleic acid synthesis and numerous roles in cell metabolism and regulation such as a cellular energy source, phosphate

group supply for phosphorylation, cofactor of enzymes and proteins. In consequence, the increase in NTP-associated gene expression observed through the larval period would suggest an intensification of cell metabolic processes in larvae that likely occurred during the transition between larval and juvenile stages.

4.10. Digestion

The expression of genes clustered in this group gave us an idea of sea bass digestive system development. Genes included those coding for digestive enzymes involved in carbohydrate [*pancreatic alpha amylase* (*AMY2A*)] and protein [*trypsin I precursor* (*PRSS1), III precursor* (*PRSS3*) and *gastricsin precursor* (*PGC*)] digestion. Genes coding for basic proteolytic enzymes (trypsins) displayed an increasing pattern of expression from 7 until 17 dph, and amylase expression decreased from 17 dph onwards. Similar results for trypsin (Srivastava *et al.*, 2002; Murray *et al.*, 2004; Darias et al., 2007a) and amylase (Cahu & Zambonino-Infante, 1994; Tanaka et al.,1996; Douglas et al., 2000; Darias et al., 2006) ontogenesis have also been observed in several other fish species. Such expression patterns agree with a progressive adaptation of digestive enzymes of reared larvae to diets containing around 60% protein. It is well known that the most important step in digestive system development of a carnivorous fish is the transition from less efficient basic protein digestion in the intestinal lumen, to more efficient acidic protein digestion that becomes effective once the gastric glands of the stomach are completely functional (Zambonino-Infante and Cahu, 2001; Darias et al., 2005, 2007b). The appearance of a fully developed stomach and acid digestion is considered as the end of the transition from the larval to the juvenile stage and the beginning of adult-type feeding characteristics in fish (Govoni et al., 1986). The expression of *gastricsin precursor* revealed that sea bass larvae began to acquire the adult mode of digestion at 25 dph, in accordance with the appearance of gastric glands (Zambonino-Infante and Cahu, 2001). The decrease in expression level of trypsin precursors, combined with the most intense gastricsin expression level at 43 dph, illustrates the importance of acid digestion in sea bass.

5. Conclusion

In summary, our work is the first multi-gene study of gene expression in sea bass. This analysis revealed differentially expressed genes that are essential for organogenesis, metabolism and development. These results were gathered in parallel to the substantial changes that occur in larval morphology, metabolism and behavior to produce metamorphosed organisms. Our work constitutes a basis for future investigation of sea bass larval ontogenesis and opens up possibilities for studies focusing on genes with interesting expression patterns. Other genes, that presently lack informative annotation but which display similar expression profiles to known genes, could be involved in similar functions. Using such a strategy to learn more about the identity and role of these 'new genes', we can improve our knowledge of the elements involved in harmonious development of sea bass larvae. The identification of candidate genes is also of particular importance in reared species so as to better understand the genetic source of phenotypic variation. Comparison of gene expression patterns in other fish species will also help our understanding of the evolution of animal development. Finally, our study demonstrates that valuable results can be obtained through a large scale heterologous microarray approach when homologous chips are not available. Possibility of cross species cDNA hybridization at subdivision or closer levels could be a beneficial tool for revealing gene expression in various marine organisms of other taxa starting from the chips already available in molluscs (Jenny et al., 2007), algae (Jamers et al., 2006) and crustaceans (Wang et al., 2006).

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Tables

Table 1
The 15 most abundant gene ontology categories associated with the 1278 analyzed genes

GO Term (biological process)	GO ID	number of genes
Physiological processes	GO:0007582	687
Metabolism	GO:0008152	527
Cellular processes	GO:0009987	413
Cell growth and/or maintenance	GO:0008151	278
Protein metabolism	GO:0019538	225
Nucleic acid metabolism	GO:0006139	191
Cell communication	GO:0007154	163
Transport	GO:0006810	150
Biosynthesis	GO:0009058	147
Signal transduction	GO:0007165	124
Development	GO:0007275	117
Transcription	GO:0006350	115
Catabolism	GO:0009056	91
Morphogenesis	GO:0009653	83
Cell proliferation	GO:0008283	76

Table 1 The 15 most abundant gene ontology categories associated with the 1,278 analyzed genes

Table 2

The 4 most highly represented biological processes (GO) associated with the differentially regulated genes

GO Term (biological process)	**GO ID**	**number of analyzed genes**	**number of regulated genes**	*P-Value*
Total		774	303	
Energy pathways	GO:0007005	49	29	0.03
Digestion	GO:0007586	6	5	0.04
Organogenesis	GO:0006629	82	39	0.04
Biosynthesis	GO:0015669	147	67	0.05

Table 2 The four most highly represented biological processes (GO) associated with the differentially regulated gene.

Figures

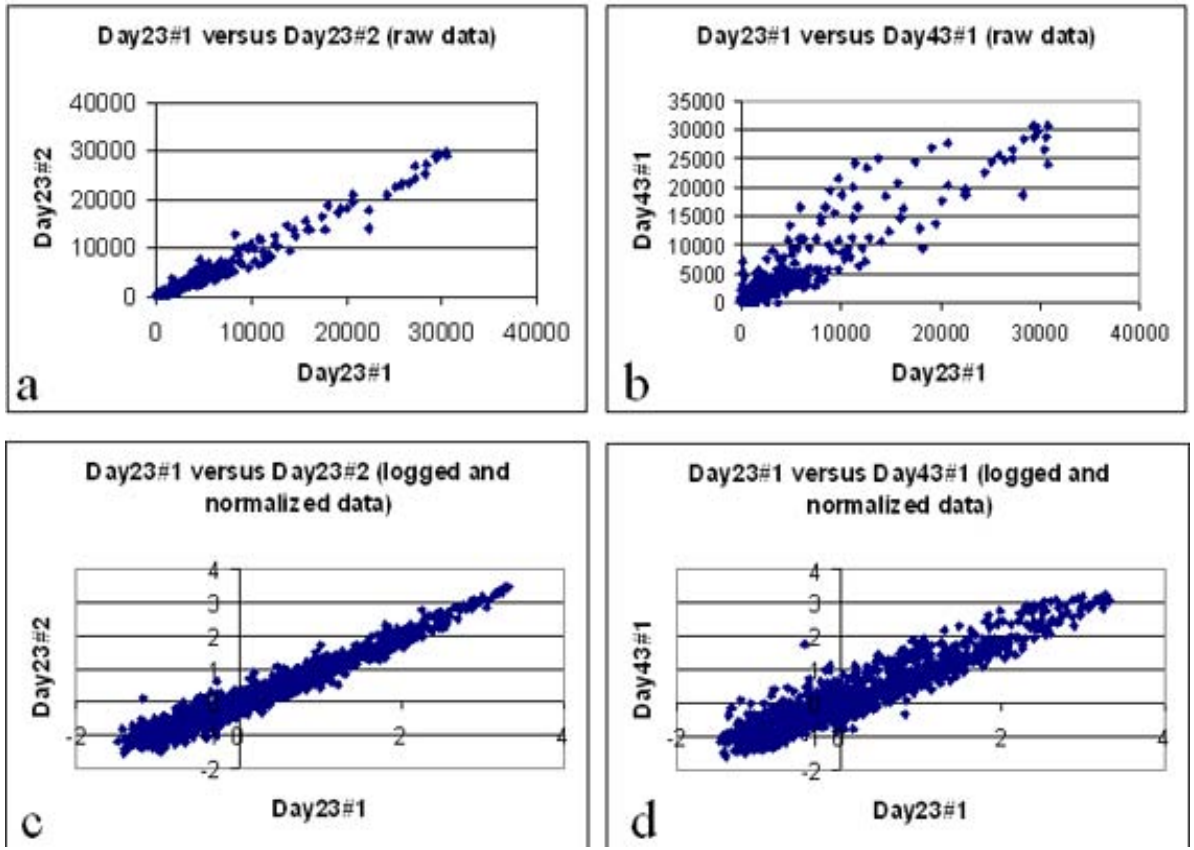

Figure 1. Effects of Log transformation and normalization (described in Materials and methods) as revealed by scatter plots. In all four figures, each axis corresponds to an experimental condition: (a and c) Day23#1 (day 23 replicate 1) versus Day23#2 (day 23 replicate 2); (b and d) Day23#1 versus Day43#1 (day 43 replicate 1). (a, b) and (c, d) show projections of the raw and logged-normalized data, respectively. Log transformation and normalization have brought the few points far away from the cloud in (a) and (b) closer to the main body in (c) and (d). Note that the cloud is narrower in (a) and (c) as compared to (b) and (d) indicating that more gene expression are regulated between day 23 and day 43 samples.

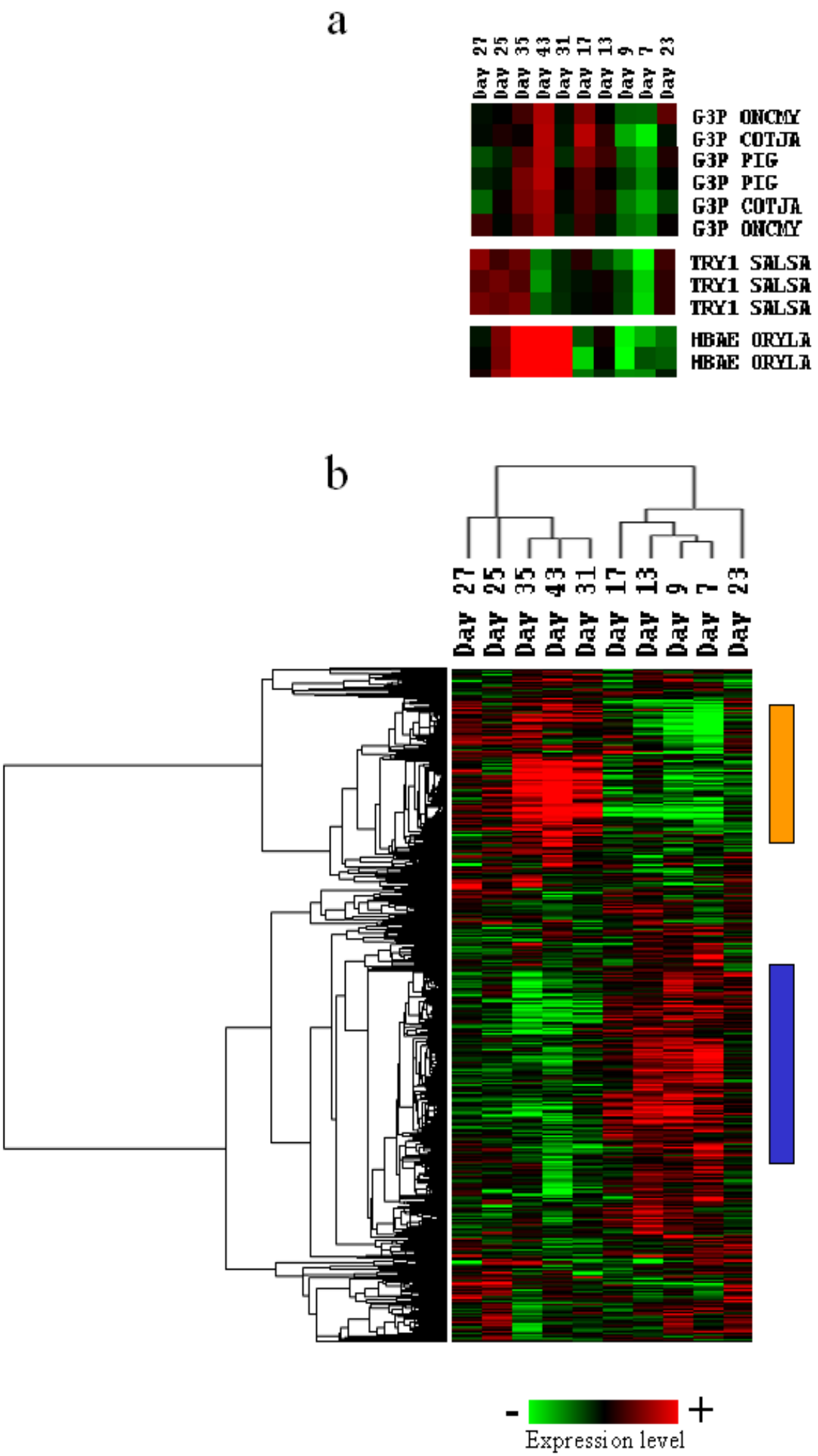
a
Day 27
Day 25
Day 35
Day 43
Day 31
Day 17
Day 13
Day 9
Day 7
Day 23
G3P ONCMY
G3P COTJA
G3P PIG
G3P PIG
G3P COTJA
G3P ONCMY
TRY1 SALSA
TRY1 SALSA
TRY1 SALSA
HBAE ORYLA
HBAE ORYLA
b
Day 27
Day 25
Day 35
Day 43
Day 31
Day 17
Day 13
Day 9
Day 7
Day 23
-
+
Expression level

Figure 2. a) Detail of the overall clustering showed a similar expression profile for different clones representing the same gene [Trypsin I (TRY1), Glyceraldehyde-3-Phosphate dehydrogenase (G3P), Hemoglobin alpha subunit (HBAE)], demonstrating the repeatability of the method. Columns represent the mean data values for each of ten time points (days post hatching) and rows represent single clones. Expression level of each gene is represented relative to its median abundance across the different stages and is depicted by a colour scale: green, black and red indicating low, medium and high relative expression levels respectively. **b)** Overall hierarchical clustering of the 1626 cDNA clones analysed based on mRNA expression level. Coloured bars to the right indicate the location of two gene clusters: blue corresponds to genes up-expressed during early stage of larvae development and orange to genes up-expressed during the late larvae stage.

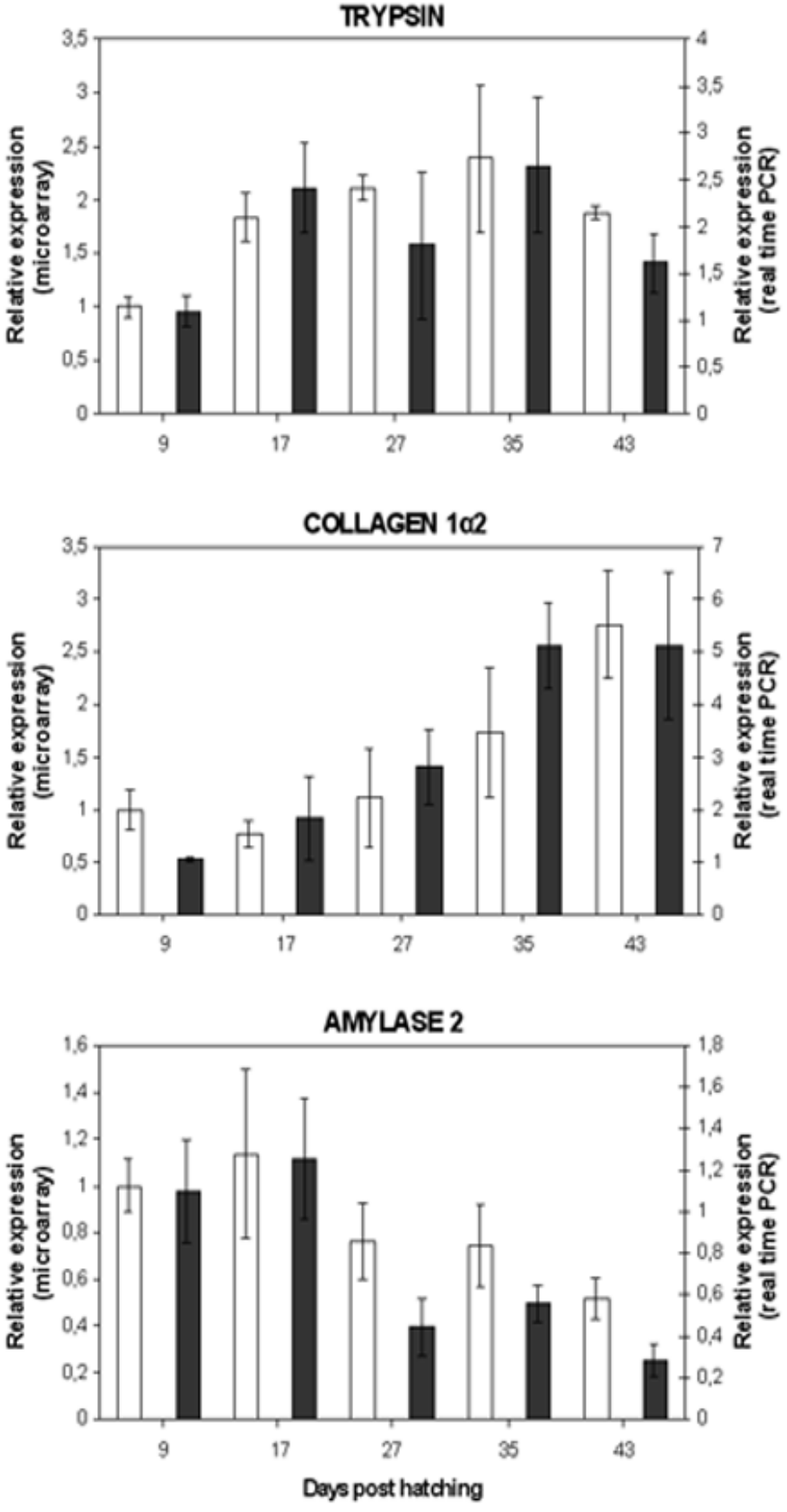
TRYPSIN
Relative expression (microarray)
Relative expression (real time PCR)
3,5
3
2,5
2
1,5
1
0,5
0
4
3,5
3
2,5
2
1,5
1
0,5
0
9
17
27
35
43
COLLAGEN 1α2
Relative expression (microarray)
Relative expression (real time PCR)
3,5
3
2,5
2
1,5
1
0,5
0
7
6
5
4
3
2
1
0
9
17
27
35
43
AMYLASE 2
Relative expression (microarray)
Relative expression (real time PCR)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
9
17
27
35
43
Days post hatching

Figure 3. Comparison between microarray (white bars) and real-time PCR (dark bars) analysis for *trypsin*, *collagen 1 alpha 2* and *amylase* mRNA relative abundance (mean ± SD) through larval development.

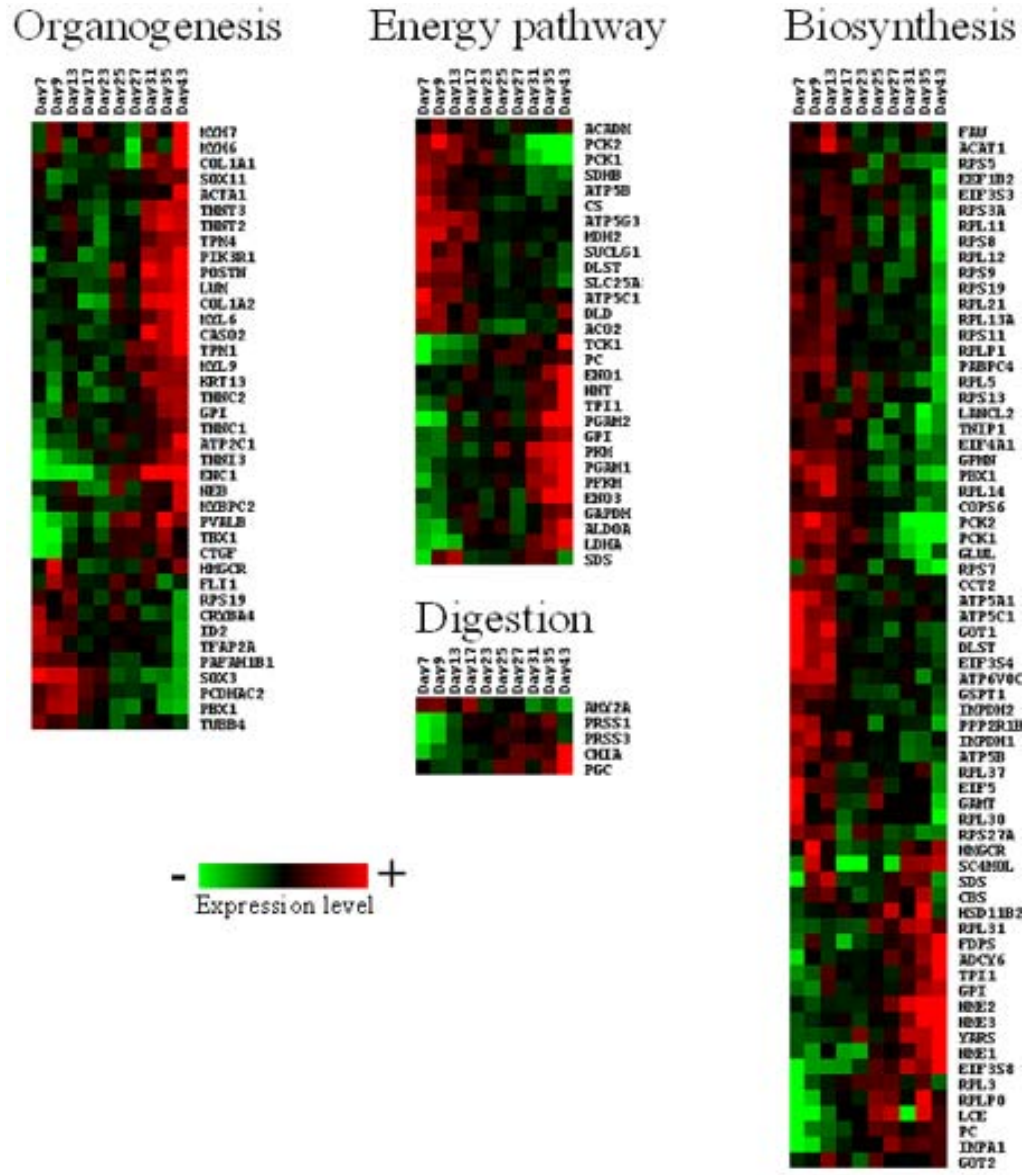

Figure 4. Expression pattern of genes involved in specific biological processes. Rows indicate gene names and columns represent the mean values of each of the ten time points (days post hatching). Genes involved in organogenesis, the energy pathway, biosynthesis and digestion were identified based on Gene Ontology (GO), and clustered separately. These biological processes were selected following EASE analysis which calculates GO over-represented in regulated genes compared with total genes analyzed.

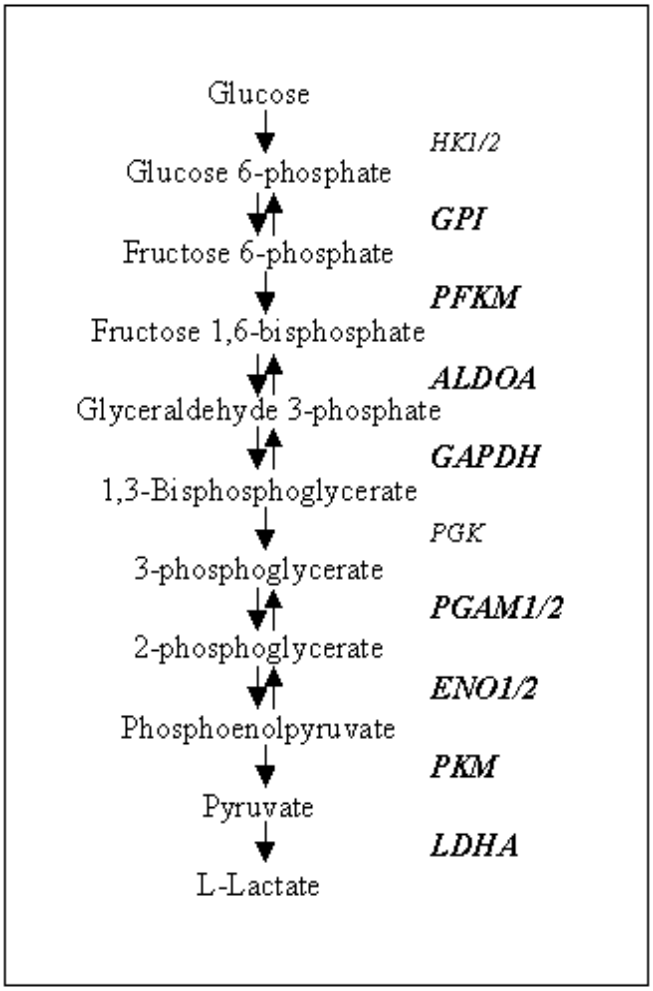

Figure 5. Glycolysis pathway from glucose to lactate. Substrates and products are written in full. The genes encoding enzymes involved in the successive reactions are indicated by their respective gene names. The genes that we found to be up-expressed during the larval growth phase are shown in bold.