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Transcriptomics for understanding marine fish larval development[1]

D. Mazurais,[a, *] M. Darias,[b] J.L. Zambonino-Infante,[a] C.L. Cahu[a]

[a] Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Technopole Brest Iroise, BP70, 29280 Plouzané, France.
[b] Investigación y Tecnología Agroalimentarias – Centre de Sant Carles de la Ràpita (IRTA–SCR), Unitat de Cultius Experimentals, Carretera del Poble Nou s/n, 43540 – Sant Carles de la Ràpita, Spain.
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* Corresponding author : D. Mazurais, email address : dmazurai@ifremer.fr

Abstract:

The larval phase is a crucial period in the life of marine fish. During this phase, the organism will acquire the phenotype of an adult fish through the development of tissues and organs and the maturation of some of the principal physiological functions. Many biological processes (differentiation, cellular proliferation, growth, etc.) are regulated during this period. These regulations take place at different biological levels and particularly concern the expression of genes involved in larval ontogenesis processes. The development of bioinformatic resources (DNA or cDNA sequences) and molecular tools enabling high throughput gene expression analysis (microarrays) have allowed the transcriptome of marine fish species to be studied. In the present review, we summarize the main findings from transcriptomic investigations of development of marine fish larvae. Special attention is paid to investigations of transcriptomic patterns during postembryonic development and to the impact of environmental or nutritional factors on the transcriptome of marine fish larvae. Transcriptomic approaches will be especially useful in the future for investigating the effect of temperature and water acidification (or pH) on the development of different fish species in the context of global climate change.

Résumé:

Le stade larvaire représente une étape cruciale dans la vie d'un poisson marin. Durant cette phase, l'organisme acquiert le phénotype du poisson adulte par le développement de tissus et d'organes et la maturation de quelques-unes des principales fonctions physiologiques. Plusieurs processus biologiques (différenciation, prolifération cellulaire, croissance, etc.) sont contrôlés durant cette période. Ces contrôles se produisent à différents niveaux biologiques et impliquent en particulier l'expression de gènes associés aux processus larvaires ontogéniques. La mise au point de ressources bioinformatiques (séquences d'ADN et d'ADNc) et d'outils moléculaires pour l'analyse à haut débit de l'expression des gènes (microréseaux) a permis l'étude du transcriptome des espèces de poissons marins. Dans notre rétrospective, nous résumons les découvertes principales des études transcriptomiques du développement des larves de poissons. Nous portons une attention particulière aux recherches de patrons transcriptomiques durant le développement post-embryonnaire et l'impact des facteurs du milieu ou de l'alimentation sur le transcriptome des larves de poissons marins. Les méthodes transcriptomiques seront particulièrement utiles dans le futur pour l'étude des effets de la température et de l'acidification (pH) de l'eau sur le développement des différentes espèces de poissons dans le contexte du changement climatique global.

1. Introduction

Fish egg hatching can take place at different embryonic stages depending on species and according to the abiotic conditions to which the eggs are subjected (the temperature, the dissolved oxygen concentration or the physicochemical composition of water) (Bagenal 1971, Bonislawska et al. 2001). After hatching, larvae of different fish species generally reach the juvenile stage by either direct or indirect development routes (Balon 1999). Direct ontogenies concern fish species such as sand-dwelling clades [*Dimidiochromis compressiceps* (Boulenger, 1908) and *Tylochromis intermedius* (Boulenger, 1916)]; these have life histories without larval stages, comparable to the development of some marine invertebrates such as Chaetognatha (Kasyanov et al. 1998). In such cases, embryos develop directly into juveniles with almost definitive phenotypes. In contrast, indirect developing fishes have an intermediate larval period between embryo and juvenile stages, during which a post-hatching organism with rudimentary organs develops into a juvenile with the definitive phenotype in most structures. As larvae grow, they develop musculature, fin rays and sensory capabilities. The striking developmental change occurring during this post-embryonic period brings about a remodelling process called metamorphosis, whose extent depends on fish species considered. The most dramatic metamorphic changes in fish are seen among flatfishes, which undergo a shift from symmetrical larvae to asymmetrical adults, including eye migration and body flattening (Ahlstrom et al. 1984; Youson 1988). Morphological aspects of metamorphosis events are less pronounced in other pelagic species. Metamorphosis is more than just a change in form, however, since this developmental change in the animal's form is associated with physiological, biochemical and behavioural changes. Indeed, metamorphosis events often coincide with migration and change of the habitat for juveniles. Therefore, larvae need to develop the physiological capacities that will enable them to face new environmental pressures at the juvenile stage, including salinity, preys and predators. Thus, whilst newly hatched fish exhibit endogenous feeding and are feeble swimmers, larvae acquire exogenous feeding capacities and their swimming capabilities improve during development, enabling future juvenile predator avoidance, feeding migrations or selective tidal transport.

It is now well demonstrated that the highest mortalities of the entire life cycle occur during larvae development, particularly during the transition from endogenous to exogenous feeding. In summary, the change from the larval to the juvenile stage requires the development of the ensemble of the organs (muscular tissue, digestive tract, central and peripheral nervous system, cardiovascular system and immune tissues) associated with a profound maturation of the principal physiological functions, and represents a critical stage in the life of a fish. It is also well documented that early life stages, particularly larval periods, are more sensitive to environmental contaminants than adult stages (Wilson 1973).

Marine fish larvae generally weigh less and are less developed at hatching than freshwater fish larvae (Houde 1994). Marine fish species thus typically have longer larval stages and suffer higher associated mortality than freshwater fish larvae. Understanding marine fish larvae development is therefore important in aquaculture in order to better define feeding sequence and general rearing conditions (current, temperature, salinity, transfer, etc.). Ontogenies of essential functions such as digestion (Zambonino-Infante and Cahu 2001, Youson et al. 2006), osmoregulation (Varsamos et al. 2005; Giffard-Mena et al. 2006), immunity (Mulero et al. 2007) and metabolism (Wieser 1995) have been extensively investigated during fish larval development (including marine species), at the level of cellular components, enzymes, metabolites and gene expression. There is indeed a consensus that regulation of gene expression is a fundamental mechanism underlying the complex processes of organogenesis, as well as the maturation of the main functions during an organism's development. Until recently, most data related to gene expression analysis during marine fish larvae development were obtained using a dedicated approach on target genes, by means of real time PCR or *in situ* hybridization.

Such investigations highlighted the importance of thyroid hormones and their receptors, diodinases genes (Isorna et al. 2009, Manchado et al. 2009) and insulin-like growth factor I (Hildahl et al. 2007*a*, *b*) in mediating metamorphosis during flatfish development. Similarly, as mentioned previously, valuable insights were also gained about the expression of genes involved in osmoregulation (Varsamos et al. 2005), digestive (Zambonino-Infante and Cahu 2001) and immune functions (Douglas et al. 2001; Mulero et al. 2007) throughout marine fish larvae development. However, these "*a priori*" dedicated approaches do not reveal the hidden aspects of processes taking place and are limiting for understanding the complexity of the regulations involved in larval ontogenesis. The recent development of genomic resources for marine fish species has provided new conceptual frameworks to better understand such regulation.
The purposes of this review are to present the benefits offered by new genomic approaches in terms of molecular tools and resources and to summarize the principal findings from recent transcriptomic investigations on marine fish larvae development.

2. Bases of the genomic approach in fish larvae development

Exhaustive approaches based on genomic tools developed on fish species over last decade have been applied to better understand the underpinning mechanisms of fish physiology, including development (for review see Douglas 2006) and the impact of different environmental or nutritional factors on embryonic and post-embryonic ontogenesis (e.g. Ton et al. 2003; Sarropoulou et al. 2005; Voelker et al. 2007; Murray et al. 2010). The complete sequencing of model fish [zebrafish *Danio rerio* (Hamilton, 1822), medaka *Oryzias latipes* (Temminck and Schlegel, 1846), pufferfish *Fugu rubripes* (Temminck and Schlegel, 1850)] genomes has greatly facilitated the genomics studies of these species.
Furthermore, genomic investigations addressing several aspects of fish physiology were initiated from the development of expressed sequence tag (EST) libraries in model species (zebrafish: Zeng and Gong 2002; Lo et al. 2003; medaka: Kimura et al. 2004; pufferfish: Clark et al. 2003) as well as in fishes of commercial interest, such as channel catfish [*Ictalurus punctatus* (Rafinesque, 1818)] (Bettini et al. 1994; Ju et al. 2000), Atlantic salmon [*Salmo salar* L., 1758] (Davey et al. 2001; Martin et al. 2002; Hagen-Larsen et al. 2005), Rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)] (Rexroad et al. 2003; Govoroun et al. 2006), Atlantic Halibut [(*Hippoglossus hippoglossus* L., 1758] (Douglas et al. 2007), Tilapia [*Oreochromis mossambicus* (Peters, 1852)] (Shiue et al. 2004), perch [(*Perca fluviatilis* L., 1758] (Rossi et al. 2007), Senegalese sole [*Solea senegalensis* (Kaup, 1858)] (Cerdà et al. 2008) or turbot [*Scophthalmus maximus* L., 1758] (Pardo et al. 2008).
The generation of EST information is an essential step in the genomic approach since it allows the identification of genes transcribed in specific organs or the whole organism. Expression profiles of the tissue from which the cDNA library was made are therefore revealed and can serve to develop markers for polymorphism. Valuable novel insights have also been obtained from cDNA libraries obtained by Suppression Subtractive Hybridization (SSH), a technique designed to identify a set of genes differentially expressed in two tissue samples when no sequence information is available (e.g. Chapman et al. 2004). Importantly, several cDNA libraries have been developed from embryonic or post-embryonic tissues or from a pool of tissues including different developmental stages, allowing the identification of sequences of genes expressed during fish development (medaka: Kimura et al. 2004; fathead minnow [*Pimephales promelas* (Rafinesque, 1820)]: Kane et al. 2008; rainbow trout: Govoroun et al. 2006; gilthead sea bream (*Sparus aurata* L., 1758): Sarropoulou et al. 2005; European sea bass [*Dicentrarchus labrax* L.,1758]: Mazurais et al. unpublished data). These results are of particularly interest, since it has been shown in vertebrates (Baldessari et al. 2005) and particularly in fish (Bai et al. 2007; Kane et al. 2008), that numerous genes involved in development are principally expressed during embryonic and larval stages, and would be probably therefore be absent from adult libraries.

The major interest of making cDNA libraries lies in their use for developing large scale EST analyses through cDNA and oligo-microarray technology. Microarrays allow large scale simultaneous measurement of relative differential gene expression between samples (from several hundred genes to whole genomes). Thus, based on functional annotation of the genes, the development of microarray technology for fish species provides a powerful tool to investigate various biological processes and molecular genetic networks involved in larval development. A method without *"a prioris"* such as this generates not only overviews of the genes and related biological processes involved in the complexity of larval ontogenesis, but allows the identification of molecular markers or candidate genes involved in the development of tissues of interest. Moreover, a high throughput approach also allows the identification of synexpression groups (groups including several genes involved in the same biological process); such results reinforce the accuracy and interpretation of gene expression data, particularly on the regulation of biological processes. Finally, an interspecific approach can be taken using these genomic tools, based on the hybridisation of labelled complex targets obtained from transcripts of a sample onto probes spotted on a support (slide or nylon). This strategy, using microarrays developed on other species for the analysis of another, has already been successfully used to investigate fish transcriptomes (Renn et al. 2004; Von Schalburg et al. 2005; Cohen et al. 2007; Kassahn et al. 2007), including fish larval development (Darias et al. 2008).

The first microarray investigations addressing fish development were performed on zebrafish embryo, which is a model system for studies of vertebrate development because it offers several advantages: a short lifespan, a large progenies, external fertilization and embryonic development, the existence of mutant lines and large panel of available microarrays (Ton et al. 2002, 2003; Voelker et al. 2007; Xiong et al. 2008).

Only a few studies have been realised on larval stages of marine fish species. Some of these studies addressed a global analysis of gene expression over the course of larval development (Sarropoulou et al. 2005; Darias et al. 2008; Douglas et al. 2008), whereas others investigated the impact of environmental or nutritional factors on the larval transcriptome (i.e. Roling et al. 2006; Connon et al. 2009; Murray et al. 2010). Main results obtained from these studies will be summarized in the following chapters.

3. Transcriptomic patterns during marine fish larvae development

3.1.1. Overview and methodological aspects

Until now, panoramic time-course analyses of transcript expression during larval development have been carried out in only three marine fish species: gilthead sea bream (Sarropoulou et al. 2005), European sea bass (Darias et al. 2008) and Atlantic halibut (Douglas et al. 2008). Depending on the species, transcriptome analysis was performed during different windows of time during larval development, always from RNA extracted from pool of whole larvae. In each case, stages were chosen that represented different developmental events, in order to obtain an overview of genes and related biological processes involved in key steps of larval ontogenesis.

In gilthead sea bream, early larval development was singled out, since larvae were sampled immediately after hatching, at 2 days post-hatching (2 dph, development of the eye and budding of pectoral fin), 4 dph (developed eye, mouth opening) and 5 dph (pigmentation of the eyes) (Sarropoulou et al. 2005). In Atlantic halibut and European sea bass, samplings were performed throughout larval development: at hatching, 21 dph (mouth opening), 64 dph (midway to metamorphosis), 91 dph (pre-metamorphosis) and 104 dph (post-metamorphosis) for Atlantic halibut (Douglas et al. 2008) and at 7 dph (pigmentation of the eyes and beginning of exogenous feeding), 9 dph, 13 dph (developed liver), 17 dph (exclusive exogenous feeding), 23 dph (beginning of vertebrae ossification), 27 dph, 31 dph, 33 dph, 35 dph (developed impaired fins) and 43 dph (adult morphology) in European sea

bass (Darias et al. 2008). The large variety of biological processes associated with the annotated genes found to be differentially expressed between time points, combined with consistencies of results obtained in different species, reveals the complexity of the transcriptome regulation during larval ontogenesis. For instance, during the early development of gilthead sea bream, more than two hundred genes involved in visual pigmentation, metabolism, digestive function and epithelial or muscle development have been shown to exhibit differential expression (Sarropoulou et al. 2005). In Atlantic halibut, 90 genes involved in transport, metabolism, lens development, signal transduction, protein degradation, apoptosis, muscle and nervous system development and function, digestive function or RNA processing were found to be regulated throughout larval development (Douglas et al. 2008). Finally, in European sea bass, more than four hundred genes involved in several hundreds of biological functions have been shown to exhibit differential expression during the 43 days post hatching (Figure 1A) (Darias et al. 2008). Considered together, these results confirm that the regulation of gene expression is a key mechanism underlying larval ontogenesis. This high number of differently expressed genes can also be explained by the fact that the analyses were carried out from whole larvae samples that included a mixture of organs and different cellular types. In samples of this type, the molecular regulation controlling larval ontogenesis concerns a broad panel of biological processes. Indeed, the mechanisms governing organogenesis in multicellular organisms depend on the regulation of genes involved in several cellular processes, such as cell proliferation, differentiation or migration, as well as other biological pathways such as protein biosynthesis, RNA processing or chromatin remodelling in different organs (Baldessari et al. 2005). However, regardless of which marine fish species is considered, the predominant changes in gene expression during larval development revealed by microarray experiments reflect processes related to organogenesis (muscular development, visual and neural development, ossification), the maturation of essential physiological functions such as digestive function and the regulation of metabolic pathways (Table 1).

3.1.2. Muscular development

Genes related to muscle development (e.g. both light and heavy chains of myosin and tropomyosin) were found to be up regulated around the time of hatching in sea bream, compared with neurula and mouth-opening stages (Sarropoulou et al. 2005). This data is in agreement with that obtained in zebrafish (Ton et al. 2002) and may be related to stratified hyperplasia, which is the main mechanism responsible for the increase in the number of muscle fibres in the late embryo and during fish larval development (Johnston, 2006). This process corresponds to the formation of muscle myotubes at the dorsal and ventral margins of the myotome, contrary to somitic myogenesis dependent on the scaffold of adaxial cell-derived slow fibres occurring during the earlier steps of development (Johnston, 2006). However, the phase of stratified hyperplasia does not start at the same time for all fish species. In fry resulting from large eggs, as in salmon, the recruitment of new myofibres starts before hatching, whereas in larvae resulting from small eggs (marine fish species such as sea bream or sea bass) the recruitment of new fibres occurs with mouth opening. Thus, in sea bream, Mascarello et al. (1994) described that if several layers of mitochondria-poor myofibril-rich deep muscle fibres covered by a superficial monolayer of mitochondria-rich myofibril-poor fibres were present at hatching, a second phase of myofibre formation occurred from mouth-opening stage when fish were reared at 15 °C. The apparent contradiction between this finding and expression data obtained by Sarropoulou et al. at mouth-opening stage may be explained by the different rearing temperatures used in these different studies (15 °C for Mascarello et al. 1994; 20 °C for Sarropoulou et al. 2005), which impacted the kinetics of development and the delay between the expression of the genes and the appearance of the tissue structure. Works from Scapolo et al. (1988) have clearly demonstrated in sea bass that muscular development during larval development was associated with the transition of myosin chains from embryonic myosin chain isoforms to

larval myosin chain isoforms between 10 and 28 days. This finding agrees well with transcriptomic data obtained in sea bass, which indicated an increase in myosin light chains (MYL6 and MYL9) as well as troponin and tropomyosin gene expression from days 25-31 post hatching (Figure 1B) (Darias et al. 2008). Similarly, approaches using molecular or immunohistochemical approaches in flatfish, such as flounder, showed that the metamorphosis process includes complex gene regulation related to muscle development (Yamano et al. 1994). Accordingly, using a high throughput approach, Douglas et al. (2008) demonstrated an increase in myosin light and heavy chains gene expression between mid-metamorphosis period and pre-metamorphosis stage. The development of the muscular tissue at the beginning and throughout the larval period can be associated with the swimming activity after hatching. Locomotive performance is indeed necessary for fish larvae to catch preys for feeding and to escape from predators.

3.1.3. Visual and neural development

The eyes of fish larvae develop indirectly, being often slightly pigmented and non-functional at hatching. The development of a functional visual system is also necessary for successful passage through the early larval stages (Falk-Petersen 2005). In fact, because most larvae are visual predators (Blaxter 1986), development of visual acuity is essential for feeding during this early stage of development. Moreover, work by Fuiman et al. (2006) identified visual responsiveness as the primary determinant of success in predation trials with fish larvae. Interestingly, Sarropoulou et al. (2005) demonstrated that many genes associated with visual development, such as gamma-crystallin M2-1, green-sensitive opsin or arrestin, exhibited up regulation just after hatching. Similarly, in sea bass or in halibut, genes related to the ontogenesis of the visual system, such as transcription factor AP-2-alpha that is required for morphogenesis of the lens vesicle (West-Mays et al. 1999), and crystallins A4 and B1, which are the dominant structural components of the vertebrate eye lens, have been found to be up-expressed during the early stage of larval development (Darias et al. 2008; Douglas et al. 2008). It is well documented that changing crystallin expression during development may be vital for lens function, which depends on a smooth gradient of refractive index avoiding spherical and chromatic aberration (Sivak and Kreuzer 1983). The fully pigmented and functional eyes observed in most marine fish species within the first week after hatching thus result mainly from the regulation of genes related to visual function during early development of marine fish species, as revealed by transcriptomic experiments. The sensory perception of environmental stimuli is not only dependant on eye formation but also on sensory neural system development. Interestingly, it has been shown that up expression of genes implicated in development of the visual system in European sea bass were associated with up expression of genes involved in sensory neural development during the early stages of larval development. The genes for platelet-activating factor acetylhydrolase IB subunit alpha, involved in proliferation and migrations of neurons (Assadi et al. 2008); protocadherin-alpha, implicated in the development of serotonergic projections (Katori et al. 2009); and *sox3*, known to regulate both neural fate and differentiation in zebrafish (Dee et al. 2008), have indeed been shown to be up regulated within the ten days post hatching (Darias et al. 2008). Furthermore, other genes involved in neural development (e.g. Ectoderm-neural cortex 1 protein, *sox11*) have also been shown to be up-regulated later on (from days 20-30 post hatching) during larval development in sea bass (Darias et al. 2008). Similarly, in halibut, Slit homolog 2 gene, involved in axonal navigation and projection of axons during neural development (Miyashita et al. 2004), was up expressed from the pre-metamorphic stage (Douglas et al. 2008). Such microarray data are consistent with results obtained in zebrafish larvae describing the development of peripheral sensory structures (Sapède et al. 2002) and post embryonic neural proliferation in the brain (Wullimann and Puelles 1999).

3.1.4. Ossification

Marine fish species hatch much earlier in their development than other vertebrates and it has indeed been shown that larvae of sea bream and sea bass are not ossified at hatching (Faustino and Power 1998; Darias et al. 2010*a*). Although the sequence of formation of bone structures is very variable from species to species, the ossification process is vital to providing a solid multifunctional structure for the organism (for locomotion, protection, support, etc). Accordingly, microarray experiments performed in sea bass revealed that transcripts related to genes involved in bone development exhibited increases in relative levels throughout larvae development and specially from 20 dph (Figure 1B). In this species, Collagen alpha 1(I) and 2(I) chain precursors, which form the fibrils of tendons, ligaments and bones; the connective tissue growth factor precursor, which promotes the proliferation and differentiation of chondrocytes; and periostin, which plays a role in the recruitment and attachment of osteoblast precursors (Horiuchi et al. 1999), are all up expressed from day 30 post hatching. This finding is in total agreement with data obtained by histological coloration, which revealed the first signs of vertebral column ossification/mineralization occurring around 30 dph in sea bass, while craniofacial skeleton ossified earlier (10 dph) (Darias et al. 2010*a*).

3.1.5. Digestive function

Feeding is essential for survival of fish larvae, especially after yolk resorption. Together with prey detection and capture capacity (which rely on visual and muscular development), a fully developed digestive tract must be acquired in several weeks or months (depending on species) after the onset of the exogenous feeding for most of marine fish larvae species. Digestive tract development involves functional maturation of pancreas, intestine and stomach, including morphological changes combined with cell differentiation. The consequence of these changes is the production of specific enzymes in the different key organs of the digestive tract. A better understanding of gastrointestinal ontogeny is particularly important for aquaculture species, in order to feed larvae with appropriate diets. Interestingly, microarray experiments performed in sea bass, sea bream and halibut indicated regulation of numerous genes associated with digestive tract maturation. In summary, data obtained in sea bass and sea bream revealed high expression of amylase during early larval development. This result is in agreement with amylase-specific activity measured in different marine fish species (Zambonino-Infante and Cahu 2001). This higher expression of amylase gene in sea bass and sea bream may be a reflection of digestive system ontogeny needed to acquire the enzymatic machinery characteristic of carnivorous fish, or a reflection of nutrient requirements at this stage of development. An increasing pattern of expression corresponding to proteolytic enzymes such as trypsin, observed from mouth-opening in halibut and sea bass, agrees with a progressive adaptation of digestive enzymes to high protein food in carnivorous fish. All together, those microarray results confirm the idea that digestive maturation in marine fish larvae corresponds to the transition from a less efficient basic protein digestion in the intestinal lumen to a more efficient acidic protein digestion that becomes effective once the gastric glands of the stomach are completely functional (Zambonino-Infante and Cahu 2001). In agreement with this pattern, the microarray results in sea bass revealed up regulation of gastriscin from 25 dph, which corresponds to the appearance of the gastric gland in this species (Zambonino-Infante and Cahu 2001; Darias et al. 2008).

3.1.6. Metabolic pathways

In the same way that behavioural, feeding and morphological changes occurring during larvae development are related to one another, microarray experiments also indicated differences in transcript levels of genes involved in metabolic pathways. Data obtained during larval development in sea bass provide a good illustration of the evolution from an aerobic

production path (Tricarboxylic Acid cycle, TCA) associated with active tissues (liver, intestine) to an anaerobic production of energy directly related to the development of white muscular tissue and resulting swimming activity. Indeed, gene expression patterns revealed an increase of transcript levels of genes involved in glycolytic pathways (glyceraldehyde 3-phosphate deshydrogenase in sea bass and halibut; Fructose-bisphosphate aldolase A, pyruvate kinase, alpha and beta enolases, phosphoglycerate mutase 1 and 2, 6 phosphofructokinase muscle type, triose phosphate isomerase, L-lactate dehydrogenase A chain, glucose-6-phosphate isomerase in sea bass, Figure 1-B) throughout larval development. While overall expression of genes involved in glycolysis increased throughout development, eight genes related to TCA exhibited higher amounts of transcripts within whole larvae from day 7 to day 17 post hatching (Darias et al. 2008). These transcriptomic results are in agreement with previous data obtained by a biochemical approach, indicating an increase in the anaerobic power of fast muscle fibres during the transformation from fish larvae to juvenile stage and an almost entirely aerobic energy metabolism in embryos and early larvae (Wieser, 1995).

3.1.7. Co-expressed gene clusters

Importantly, large scale gene expression screens can reveal the co-ordinate expression of genes. The determination of co-expressed gene clusters allows genes to be assembled into common or connected molecular pathways and the role of genes of unknown function to be predicted. In cDNA microarray experiments, such coordinated changes are obvious in so-called expression clusters, groups of genes whose expression profile is more or less tightly correlated (Eisen et al. 1998). Cluster analysis performed during sea bass development showed two major phases of coordinate gene expression between hatching and late larval development (Figure 1). The first phase covers stages from day 7 to 23 post hatching, during which genes involved in visual and neural development, as well as genes of aerobic metabolism, are more greatly expressed overall within whole larvae. During the second phase, from 25 to 43 dph, there is higher expression of genes involved in ossification, muscular development, anaerobic metabolism and digestive tract maturation. The period from 17 dph to 30 dph corresponds to a shift in transcriptomic pattern during sea bass larvae development and is associated with the maturation of some major physiological functions already described in previous studies (Zambonino-Infante and Cahu 2001) and is probably an essential step in the gradual process of metamorphosis.

4. Impact of environmental or nutritional factors on the fish larvae transcriptome

As we have illustrated, adequate development depends on the regulation of a succession of biological processes operating at different developmental stages. It also depends on molecular and cellular mechanisms that buffer organisms from expected environmental stresses (Hamdoun and Epel 2007). However, these adaptive capacities set physiological limits and embryos and/or larvae exhibit abnormal development when stress exceeds these limits. As the transcriptome plays an important role in the regulation of biological processes and the resulting phenotype, large scale gene expression analyses have been performed in order to better understand the impact of environmental stress on physiological functions, as well as to indicate molecular markers associated with biological dysfunctions. Although several studies have been made on the impact of stressful environmental or nutritional factors on fish transcriptomes during recent decades, particularly in the field of toxicogenomics (for reviews, see Ju et al. 2007; Prunet et al. 2008), only a few have employed a microarray approach to analyse effects of external factors (pollutants, feeding, global change) on marine fish larval development.

4.1. Toxicogenomics

Several experiments have been performed specifically in the field of toxicogenomics of fish, since aquatic species are frequently used as models to characterize the effects of environmental pollutants. The interest of the transcriptomic approach lies in the fact that changes in gene expression are useful biomarkers of toxicant exposure, providing information about an organism's health, adaptability and toxicant-specific reactions. Special attention has been paid to the impact of pollutants on embryonic and larval development since early life stages are very sensitive to environmental contaminants. In zebrafish, which represents a model commonly used in the field of ecotoxicology (Hill et al. 2005; Scholz et al. 2008), several gene expression analyses have been performed at embryonic stages to analyse the effects of toxicants (Hoyt et al. 2003; Carney et al. 2006; Xu et al. 2006; Kreiling et al. 2007; Xiong et al. 2008). The interest of such investigations has been particularly pointed out by Voelker et al. (2007) who demonstrated that high throughput analysis of gene expression is capable of revealing mechanistic information that cannot be obtained by the classical endpoints used in the zebrafish embryo toxicity test. However, as indicated previously for overall gene expression pattern throughout the course of larval development, only a few toxicogenomics studies have been carried out on larval stages in marine fish species. In one of them, the molecular mechanism associated with growth inhibiting effects of hexavalant chromium were monitored in mummichog, Fundulus heteroclitus (Roling et al. 2006). After 30 days of exposure, hybridization of a macroarray system with 233 unique genes, revealed that 18 genes were significantly regulated in a dose-dependant manner, many of these regulated genes were involved in toxicant responses (Aryl hydrocarbon Receptor), energy metabolism (Glucose transporter GLUT2, Fatty Acid Binding Protein), structure (type II keratin) and the immune system (Complement components C9 and C3-2). Such expression measurements, even if obtained from low density array, provide clues about the mechanism for the growth-reducing effects of chromium during larval development.
In delta smelt [*Hypomesus transpacificus* (McAllister, 1963)], whose populations are in decline in the freshwater-saltwater mixing zone of Californian estuaries, the impact of the commonly-used insecticide ensfenvarelate on transcriptomic pattern was investigated using a microarray with 8448 cDNA fragments (Connon et al. 2009). Statistical analysis revealed that low concentrations of the pollutant caused regulation of 118 annotated genes involved in about 15 biological processes including neuromuscular activity processes (19% of regulated genes). Interestingly, regulation of several neuromuscular genes could be related to swimming impairments resulting from pollutant exposition.
An additional application of the microarray approach in the investigation of toxicant impact is its use to screen and identify molecular markers that can be directly used in endangered organisms. Such toxicogenomic analysis should now be applied to other compounds with different chemical properties and modes of action to obtain a panel of biological markers that will enable us to diagnose the exposition and evaluate the impact of several pollutants on marine fish larvae development.

Nutrigenomics

In the context of aquaculture, it is well documented that high mortality during the early stages of larval development can be linked to the shift from endogenous to exogenous feeding and more particularly to nutrient deficiencies (Zambonino-Infante and Cahu 2007).
In flatfish species, poor performance at the start of feeding has been shown to affect successful metamorphosis, particularly eye development and migration, skeletal development and skin pigmentation (Naess and Lie, 1998; Hamre et al. 2005, 2007). Murray et al. (2010) investigated the impact of replacing live prey (Artemia) with microencapsulated

diet on Atlantic halibut development, using a combination of morphometrics and histological approaches combined with transcriptomic analysis. Interestingly, results showed that the introduction of this microdiet 20 days after the start of feeding disrupted growth and metamorphosis processes (eye and skin pigmentations) and that these dysfunctions were associated with the modified expression of fifty eight genes. Among the regulated genes, some structural proteins (Titin N2-B, Collagens Ia3 and Ixa1) were down expressed following the introduction of the microdiet; a change which would result in lower growth. Genes involved in eye development (Crystallin beta A2-2, protein similar to es1, peripherin1) or skin pigmentation (Transmembrane protein 33) were up-regulated in fish fed the microdiet. These regulations could contribute to some of the phenotype abnormalities seen in the larvae.
In European sea bass, numerous studies have demonstrated the influence of dietary vitamins on growth, survival, and morphogenesis during larvae development (Mazurais et al. 2008, 2009) and notably the impact of vitamin D3 levels (Darias et al. 2010*b*). A dedicated approach based on qPCR analysis revealed the effect of dietary vitamin levels on the expression of vitamin receptors (such as retinoic acid or vitamin D receptors). It is generally accepted that regulation of these receptors, which are members of the nuclear receptor family of transcription factors, should regulate the expression of downstream genes involved in ontogenic pathways. Interestingly, a microarray approach recently showed that growth and morphogenesis perturbations observed in sea bass larvae fed vitamin D3-deficient diet were associated with the regulation of several genes involved in cell proliferation and differentiation, particularly in nervous system development (Darias et al. unpublished data).

Although the nutrigenomics approach is still in its infancy, particularly for fish developmental stages, in the future it should lead to an improved understanding of how nutrition influences biological processes, including metabolic and ontogenic pathways.

Perspectives for the investigation of environmental factors

In the environmental context of global climate change, the adaptive response to environmental stress is of great importance in marine species. Although the impact of these environmental cues has been extensively investigated over the last decade using histological, biochemical and dedicated molecular approaches on fish of ecological or economic interest, very few studies have been performed in the field of genomics especially for larval stages in marine fish species. In contrast, the impacts of environmental factors such as hypoxia have been monitored on the transcriptome of model species such as zebrafish or medaka (Ton et al. 2003; Van der Meer et al. 2005; Boswell et al. 2008) at embryonic or adult stages, as well as in adult common carp (*Cyprinus carpio* L., 1758) (Fraser et al. 2006) and goby (*Gillichthys mirabilis* Cooper, 1864) (Gracey et al. 2001). Altogether, those studies showed that microarray analyses are well suited for studying responses to low oxygen levels, particularly specific changes in gene expression enabling us to identify mechanisms for adaptive response to hypoxia. Such approaches will be necessary in the future to better understanding the impact of hypoxia stress on the biological processes underlying marine fish larvae development and more particularly to compare the response of species exhibiting different capacity to withstand long exposure to hypoxia.
Similarly, it will be informative to investigate the effects of all environmental cues associated with global climate changes such as temperature, water acidification or pH on the transcriptome of whole larvae to better understand the overall physiological impact of such factors. Indeed, it has been shown in several previous studies that such factors, including for example a small rise in water temperature, could dramatically regulate several functions or behaviours in different marine fish species (reviewed in Roessig et al. 2004).

Conclusion: optimisation of genomic approaches for fish larvae physiological studies

Despite the very informative results obtained with the transcriptomic approach in fish, the lack of reliable annotation for a large number of ESTs in fish libraries remains a limiting factor in the interpretation of the data (e.g. 50 % in Atlantic salmon, Hagen-Larsen et al. 2005; 35 % in catfish, Wang et al. 2010). Moreover, when annotation is available, it can be inadequate, depending on the hit score, or liable to frequent change with new EST clustering and database releases. Sequence annotation will improve in the future as the genome sequence becomes available for some marine fish species. The acquisition of new annotated sequences will also aid the proteomics approach in marine fish species, which faces the problem of annotation of peptides of interest.

Another limiting factor is related to the functional annotation of the genes in fish species in which the genome has been duplicated during the evolution of vertebrates. While the vertebrate genome is a result of two rapid and successive rounds of genome duplication, one proposed mechanism suggests that a third whole genome duplication occurred specifically in the Actinopterygii (Hedges and Kumar 2002). While most of the duplicated genes were lost or are non-functional, some of the paralogous genes could have been retained through a process of neofunctionalization (Lynch and Force 2000). Such a characteristic could partially distort the interpretation of the microarray data for these species since functional annotations are mainly based on the characterisation of gene function in model species. Interpretation of microarray data will also be favoured by the development of high density microarrays containing oligomers, which are now available for some non model fish species (Douglas 2006). This technological advance allows, by designing adequate oligomers, to discriminate the expression of genes with similar cDNA sequences but different functions that belong to the same family as well as splicing variants of the same genes.

Finally, the last but not the least important limitation to most transcriptomic studies on fish development relates to the use of RNA obtained from whole larvae, because such samples include a mixture of various organs and cell types. The use of these complex samples hampers the interpretation of the data since most genes are expressed in several tissues, making the association of the pattern of one gene expression with a specific organ a hazardous assumption. Moreover, the interpretation of the data is also limited by the fact that the regulation of gene expression in specific tissue or cell types can be masked by the mean expression pattern throughout the other organs in the whole larvae. In the future, a better interpretation of the dataset will be possible by combining microarray data on marine fish larvae development with large scale *in situ* hybridisation, as has already been done for some model species such as zebrafish (Ouyang et al. 2008). Furthermore, dissection of specific tissues in the larvae could unravel the complexity of microarray data obtained from whole larvae samples. Indeed, it has already been shown that the organ dedicated approach can give precious and unambiguous microarray expression data, particularly concerning gonadal differentiation in rainbow trout (Baron et al. 2007). Accordingly, the development of apparatus allowing precise micro-dissection of specific tissue by a laser method and availability of kits allowing amplification of RNA obtained from very small samples will be of great utility.

Altogether, these optimisations will allow more precise and exhaustive information to be gathered concerning genes and associated physiological processes involved in larval ontogenesis of marine fish species that could be impacted by environmental and anthropic changes.

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Figures

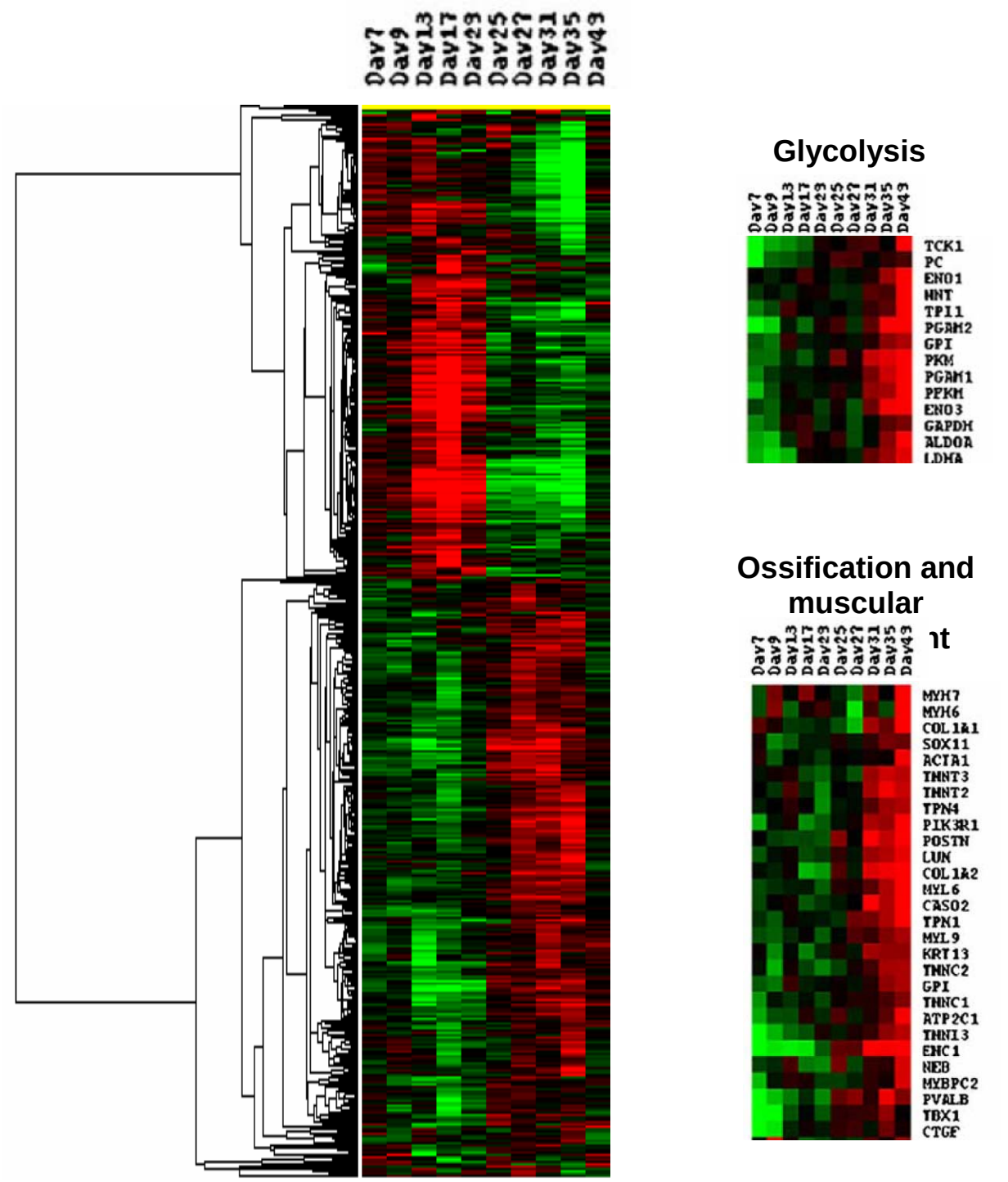

Figure 1: A- Global hierarchical clustering of more than 400 genes differentially expressed throughout European sea bass larvae development. Red and green colours indicate higher and lower relative expression of the gene in samples, respectively. B- Gene clusters related to glycolysis, ossification and muscular development. Genes are annotated with official gene names (Adapted from Darias et al. 2008).

Table 1 summarizing genes and related functions that are regulated throughout marine fish larvae development as revealed by microarray experiments

Function	species	Gene	Expression level in whole larvae	reference
Muscle development	Gilthead sea bream	*Myosin light chain 2*	increase around hatching	Sarropoulou et al. 2005
		Myosin light chain 3	"	"
		Myosin heavy chain	"	"
		Tropomyosin	"	"
	European sea bass	*Myosin light chain 6*	increase from 25-31 dph	Darias et al. 2008
		Myosin light chain 9	"	"
	Atlantic halibut	*Myosin light chain 2*	increase at premetamorphosis stage (91 dph)	Douglas et al. 2008
		Myosin light chain B2	"	"
		Myosin heavy chain	"	"
		Myosin heavy chain A3	"	"
		Tropomyosin 3	"	"
		Troponin 1-like protein	"	"
Visual development	Gilthead sea bream	*Gamma-crystallin M2-1*	increase just after hatching	Sarropoulou et al. 2005
		Green-sensitive opsin	"	"
		Arrestin	"	"
	European sea bass	*Transcription factor AP-2-alpha*	increase just after hatching	Darias et al. 2008
		Crystallin A4	"	"
	Atlantic halibut	*Crystallin B1*	increase just after hatching	Douglas et al. 2008
Neural development	European sea bass	*Platelet-activating factor acetylhydrolase IB*	increase within 10 dph	Darias et al. 2008
		SOX-3	"	"
		Protocadherin alpha C2 precursor	"	"
		Tubulin beta-4 chain	"	"
		Ectoderm-neural cortex 1 protein	increase from 20 dph	Darias et al. 2008
		SOX-11	"	"
	Atlantic halibut	*Slit homolog 2 gene*	increase at premetamorphosis stage (91 dph)	Douglas et al. 2008

Ossification	European sea bass	*Collagen alpha 1(I)*	increase from 30 dph	Darias et al. 2008
		Collagen alpha 2(I)	"	"
		Connective tissue growth factor precursor	"	"
		Periostin	"	"
Digestive function	Gilthead sea bream	*Amylase*	increase just after hatching	Sarropoulou et al. 2005
	European sea bass	*Trypsin I precursor*	increase from mouth opening	Darias et al. 2008
		Trypsin III precursor	"	"
		Pancreatic alpha amylase	decrease from 17 dph	
Metabolic pathway (glycolysis)	Atlantic halibut	*GAPDH*	increase at 64 dph (midway to metamorphosis)	Douglas et al. 2008
	European sea bass	*GAPDH*	increase from 25 dph	Darias et al. 2008
		Fructose-bisphosphate aldolase A	"	"
		Pyruvate kinase	"	"
		Alpha enolase	"	"
		Beta enolase	"	"
		Phosphoglycerate mutase 1	"	"
		Phosphoglycerate mutase 2	"	"
		6 phosphofructokinase muscle type	"	"
		Triose phosphate isomerase	"	"
		L-lactate dehydrogenase A chain	"	"
		Glucose-6-phosphate isomerase	"	"
Metabolic pathway (Tricarboxylic Acid cycle)	European sea bass	*Aconitate hydratase*	decrease from 17 dph	Darias et al. 2008
		Succinate dehydrogenase	"	"
		Succinyl-CoA ligase	"	"
		Citrate synthase	"	"
		Malate dehydrogenase	"	"
		Dihydrolipoyllysine-residue succinyltransferase	"	"
		Dihydrolipoyl dehydrogenase	"	"
		Acyl-CoA dehydrogenase	"	"