Functional Genomics in Aquaculture

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WILEY-BLACKWELL
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Editorial offices: 2121 State Avenue, Ames, Iowa 50014-8300, USA
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK
9600 Garsington Road, Oxford, OX4 2DQ, UK

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Library of Congress Cataloging-in-Publication Data
Functional genomics in aquaculture / edited by Marco Saroglia and Zhanjiang (John) Liu.
p. cm.
Includes bibliographical references and index.
ISBN 978-0-470-96008-0 (hardcover : alk. paper) 1. Fishes–Breeding. 2. Shellfish–Breeding. 3. Genomics. 4. Aquaculture. I. Saroglia, Marco. II. Liu, Zhanjiang.
SH155.5.F86 2012
639.2–dc23

2012007484

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Set in 10/12 pt Dutch801BT by Aptara® Inc., New Delhi, India

1 2012

Contents

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Preface

Genomics as a discipline has achieved more in less than 30 years in generating a vast amount of biological information than the accumulation of all biological information in history. Currently, the genetic composition of a large number of organisms has been or is being deciphered, including a large number of aquaculture species. As increased understanding is gained with the structure, organization, and evolution of genomes, scientists finally come to the point to ask some really serious questions concerning gene functions. Genomics is now undergoing a transition or expansion from the mapping and sequencing of genomes to an emphasis on genome functions. Along with the transition, the major goals of genomics shift from structural genomics to functional genomics.

Functional genomics represents a new phase of genome analysis. It requires the development of innovative technologies that make use of the vast resource of structural genomics information. Specifically, functional genomics refers to the development and application of genome-wide experimental approaches to assess gene functions by making use of the information and resources provided by structural genomics. It is characterized by high throughput or large-scale experimental methodologies combined with statistical and computational analysis of results. The fundamental strategy in functional genomics is to expand the scope of biological investigation from studying a single gene or proteins, or a few genes, to studying all genes or proteins at once on a genome-wide scale. Such operations allow the generation of tremendously large data sets that demand additional capacities of data analysis to draw information relevant to biology. Computational biology will play a critical and expanding role in functional genomics, as it is characterized by mining the data sets for valuable biological information.

Functional analyses of genomes in aquaculture include all performance and production traits such as growth, development, nutrition, sex determination, reproduction, immunity and disease resistance, and response to various environmental and physiological conditions. Initially, such genome-scale issues can be addressed through association of genome expression with the performance, and then the functions of the genes involved in specific traits can be characterized into gene pathways. Eventually, however, the functions for the vast majority of aquaculture-related traits can be approached by the combination of transcriptome profiling for expression candidates, genome-wide association studies for positional candidates, comparative genome analysis for functional candidates, quantitative genomic analysis, and finally, directly testing the candidate genes to validate their functions. Because aquaculture species involve a wide spectrum of organisms ranging from invertebrate to vertebrate animals, and many of the involved species have very special biological characteristics such as extremely high fecundity, extremely high and low tolerance to low dissolved oxygen, among many others, functional genomics research of aquaculture species will undoubtedly have a profound impact on biology.

This book has 15 chapters. The first two chapters provide a general introduction to functional genomics and general approaches for functional genomics research in aquaculture, and existing genomic resources for functional genomics in aquaculture species. Chapters 3–10 are devoted to functional genomics of growth, development, reproduction, nutrition, and responses to stress and diseases; and the last five chapters are specific chapters that

cover functional genomic studies in rainbow trout, Atlantic cod, catfish, shrimp, and molluscs.

This book covers the current status, technologies, progress, and perspectives of functional genomics in aquaculture. It should be useful to academic professionals, research scientists, graduate students and college students in agriculture, as well as to students of aquaculture and fisheries. We are grateful to all the contributors of this book. It is their great experience and efforts that has made this book possible. We have had another year of pleasant experience interacting with Ms. Susan Engelken, Editorial Program Coordinator; Anna Ehler, Senior Editorial Assistant; and Justin Jeffryes, Commissioning Editor for Plant Science, Agriculture, and Aquaculture with Wiley-Blackwell of John Wiley & Sons.

During the course of editing this book, we have worked extremely hard to fulfill our duties as professors and administrators. We would like to thank our families, particularly Genciana Terova and Dongya Gao, for their persistent support and love.

Marco Saroglia
John Liu

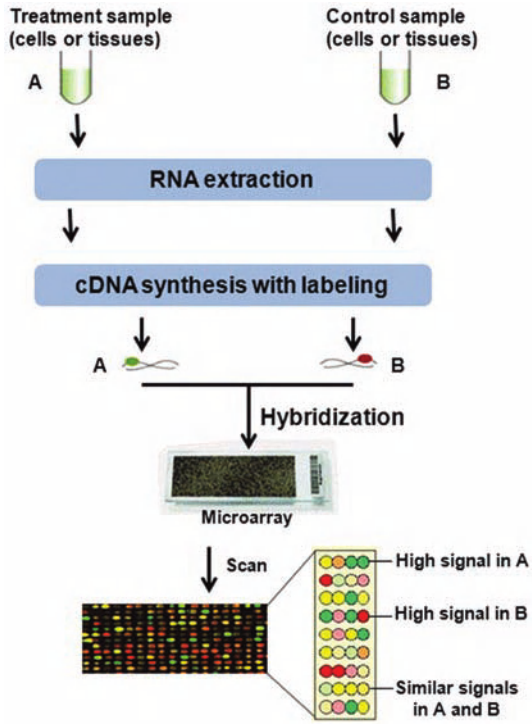


Plate 1.1 A schematic presentation of microarray experiment where expression of two samples are compared with A being the sample under treatment, and B being the sample for control. RNA is separately isolated from the samples, and fluorescently-labeled probes are made separately with different labels, e.g., cy3 for treatment (green), and cy5 for control (red). The probes are simultaneously used to hybridize an array containing the features representing the transcripts of the organism. After hybridization, signals are scanned and analyzed. If the signals are high in the treatment, green will be detected, and if the signals are high in control, red will be detected; if the signals are equal, yellow will be detected. Based on the relative signals of red and green, expression levels are determined.

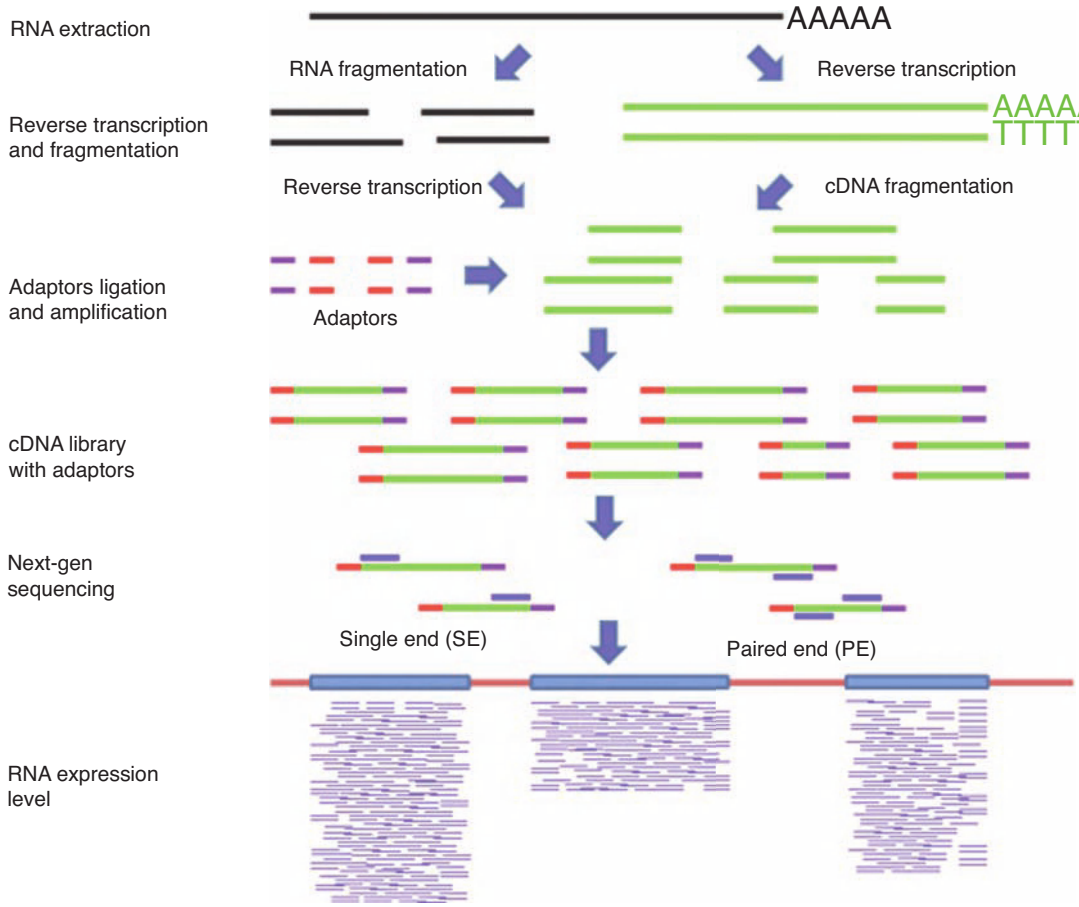


Plate 1.2 A schematic presentation of RNA-Seq. The extracted RNA is first converted into a library of complementary DNA (cDNA fragments through either RNA fragmentation (left) or DNA fragmentation (right). Sequencing adaptors (depicted by short red bars and short purple bars) are subsequently ligated to each cDNA fragment (green lines) and short sequence reads (single end or paired ends) from each cDNA are generated using high-throughput sequencing technology. The resulting sequence reads [short lines beneath the genome sequence with three genes shown (fat blue bars)] are aligned with the reference genome to evaluate gene expression by counting mapped reads. In the given example, the gene on the very left are expressed at a very high level, and the gene in the middle is expressed at a relatively lower level.

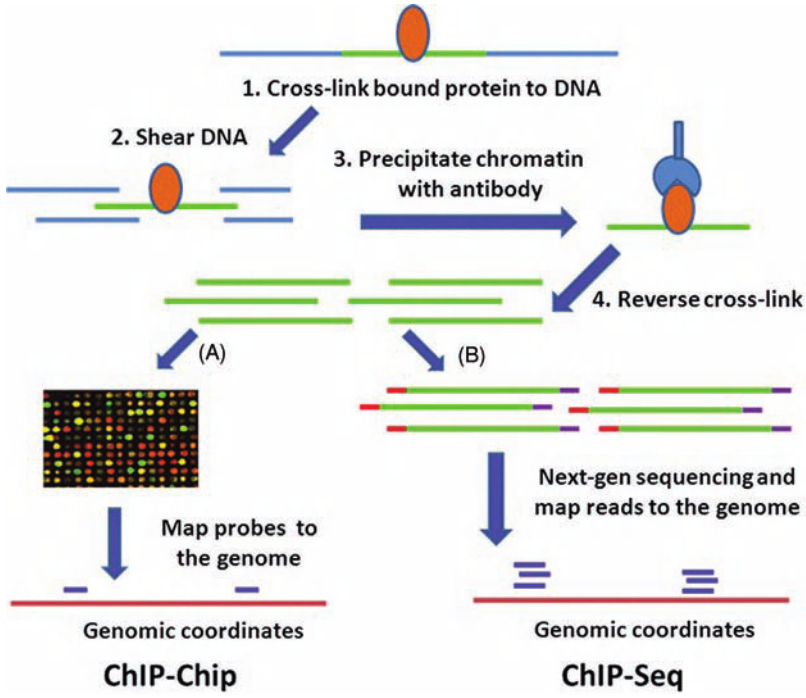


Plate 1.3 A schematic presentation of Chromatin immunoprecipitation (ChIP) combined with microarray (ChIP-Chip) and high-throughput sequencing techniques (ChIP–Seq). The first step of ChIP is to cross-link bound proteins to DNA, then isolate the chromatin and shear DNA, precipitate chromatin with protein-specific antibody, and lastly, reverse cross-link and digest protein to release the bound DNA. In ChIP-Chip (left lower panel), the ChIP DNA is then amplified to obtain sufficient DNA followed by hybridization to a DNA microarray. The microarray probes can then be mapped to the genome to yield genomic coordinates; in ChIP–Seq (right lower panel), the ChIP DNA ends are repaired and ligated to a pair of adaptors, followed by high throughput sequencing. The resulting sequence reads are mapped to a reference genome to obtain genomic coordinates that correspond to the immunoprecipitated fragments.

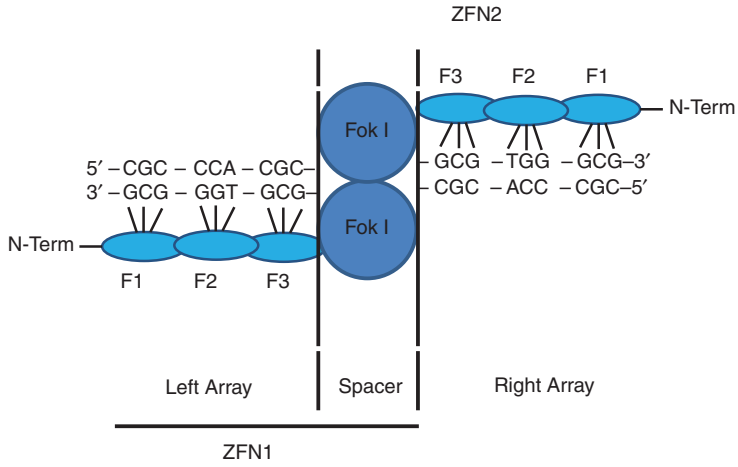


Plate 1.4 Presentation of engineered zinc finger nuclease (ZFN). The sequence specificity is determined by individual ZFN1 and ZFN2 fingers. The Fok I endonuclease domain introduces double-stranded breaks after sequence-specific DNA binding. (This figure was adapted from Dr. Stephen C. Ekker (2008) with his permission.)

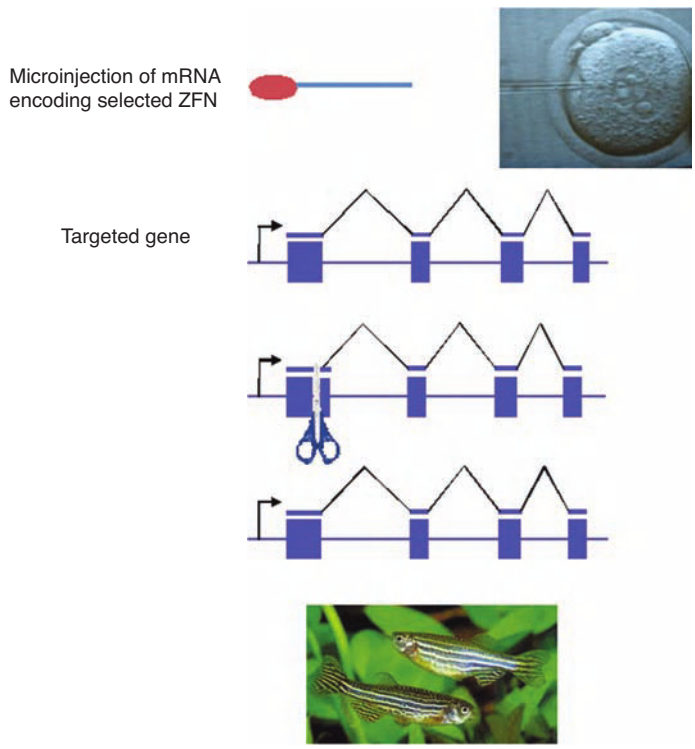


Plate 1.5 Schematic presentation of the processes of making a targeted gene knockout in zebrafish using ZFNs. mRNA encoding the selected ZFN is injected into one-cell zebrafish embryos. This custom ZFN binds and makes a double stranded break at the specified locus. Cell repairs the double stranded DNA break imprecisely to introduce mutations to the selected gene. The specific molecular lesion is clonally selected and determined after genotyping the offspring from these mosaic founders. (This figure is adapted from Dr. Stephen C. Ekker (2008) with his permission.)

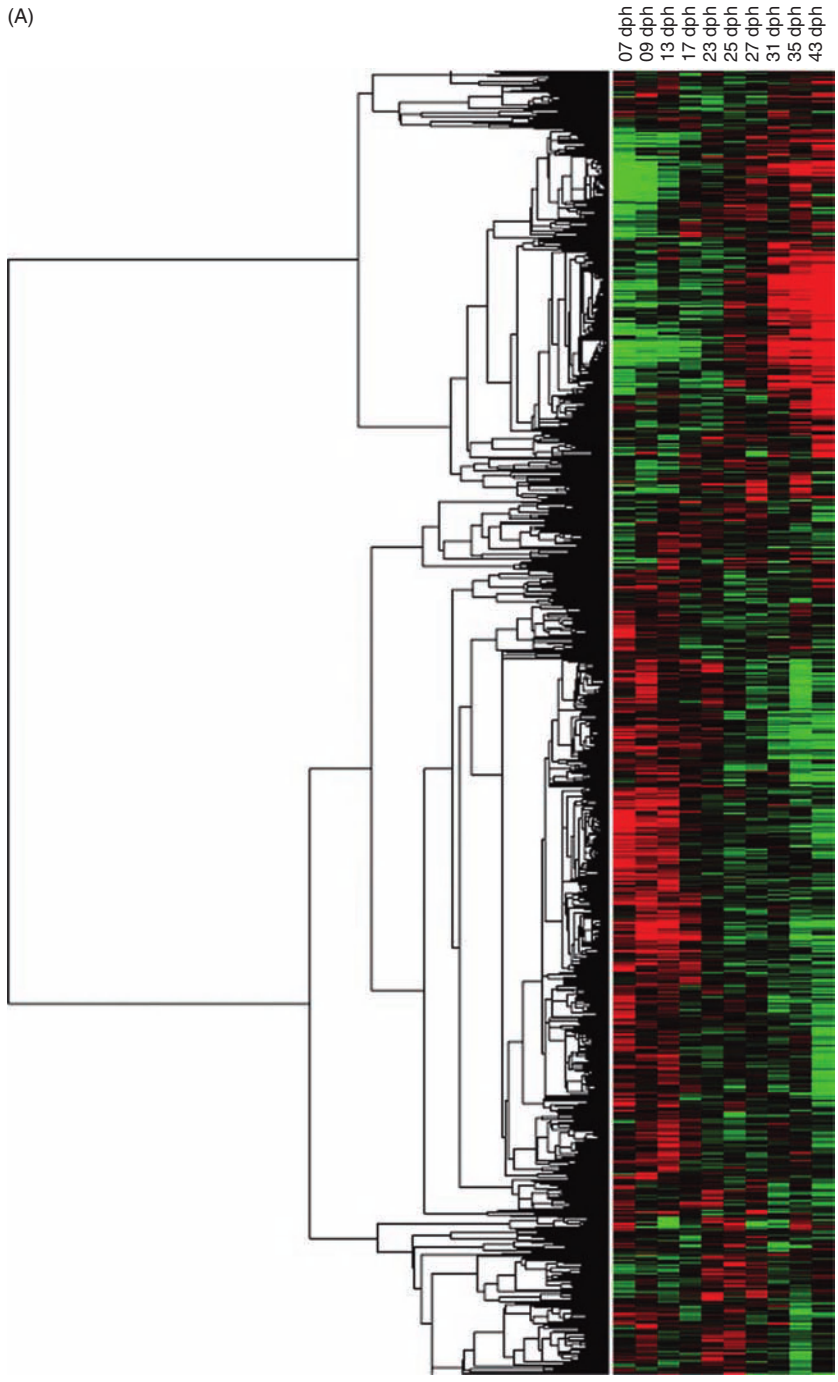
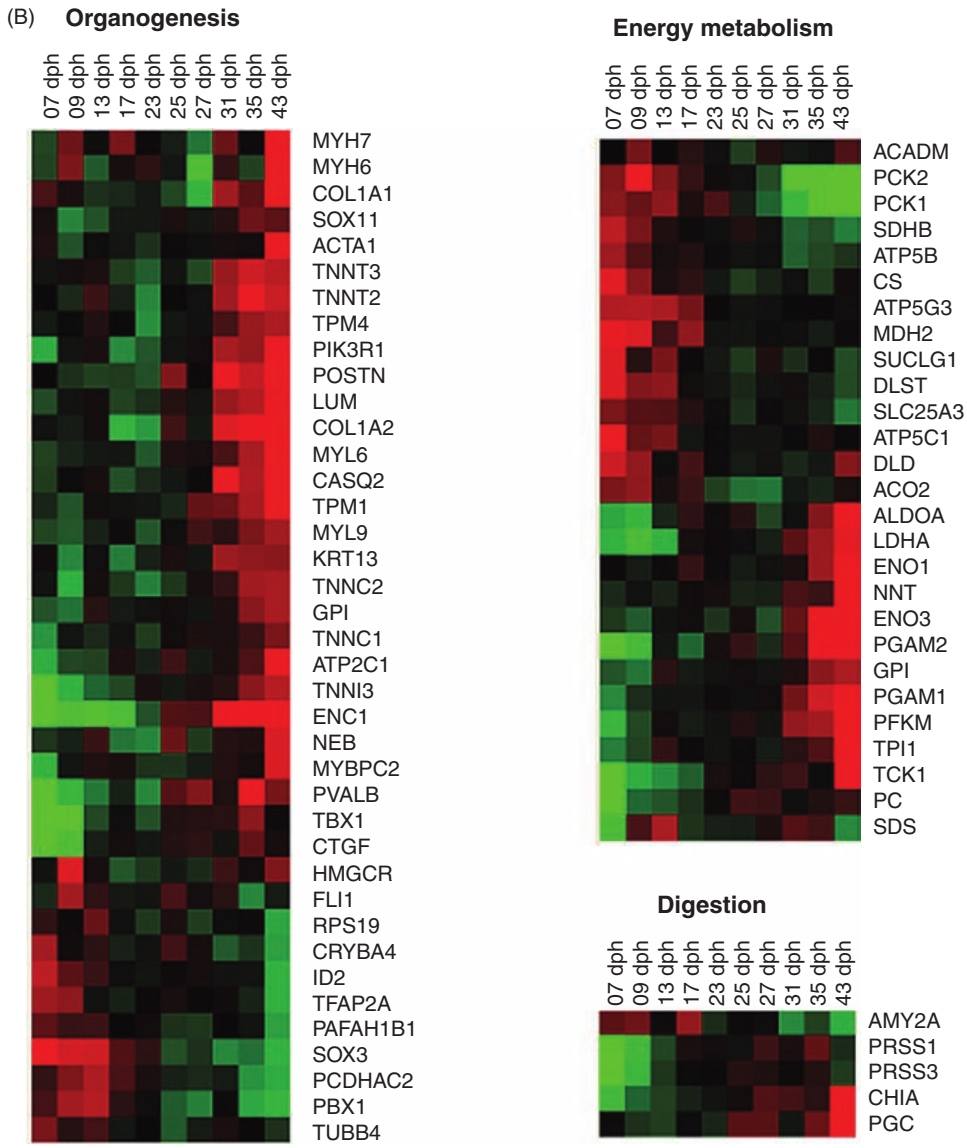


Plate 4.2 Clustering analysis of microarray studies of gene expression during early development of European sea bass. (A) Global hierarchical clustering of 1278 differentially expressed genes throughout sea bass larval development. (B) Gene clusters related to biological processes of interest. Genes are annotated with official gene names. (Adapted from Darias et al., 2008.)



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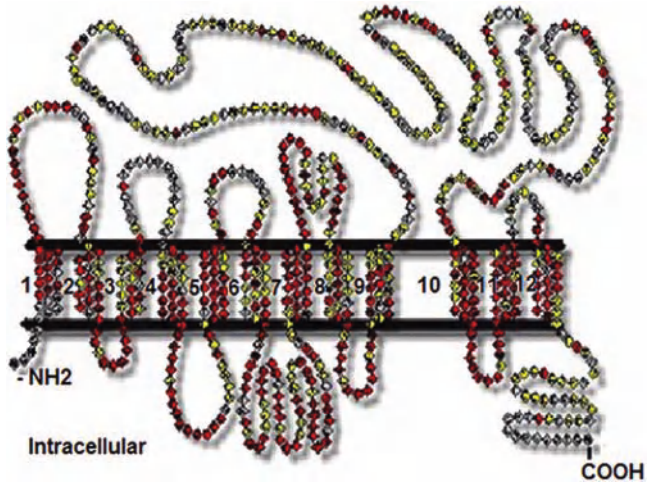

Plate 5.3 Membrane spanning helices in sea bass PepT1 protein predicted with the THMM program (http://www.cbs.dtu.dk/services/).

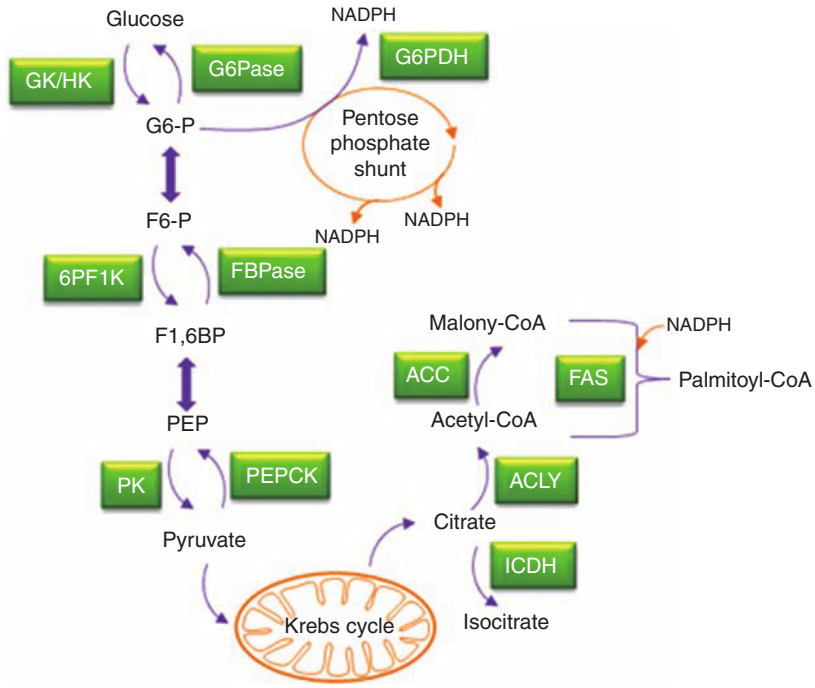

Plate 6.1 Schematic representation of metabolic pathways involved in glycolysis, gluconeogenesis, pentose phosphate shunt, and lipid synthesis. Glycolytic enzymes: glucokinase (GK), 6-phosphofructo-1-kinase (6PF1K), and pyruvate kinase (PK). Gluconeogenic enzymes: phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase). Pentose phosphate shunt enzymes: glucose-6-phosphate dehydrogenase (G6PDH). Lipogenic enzymes: ATP-citrate lyase (ACLY), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and isocitrate dehydrogenase (ICDH). G6-P, glucose-6-phosphate; F6-P, fructose-6-phosphate; F1,6BP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate.

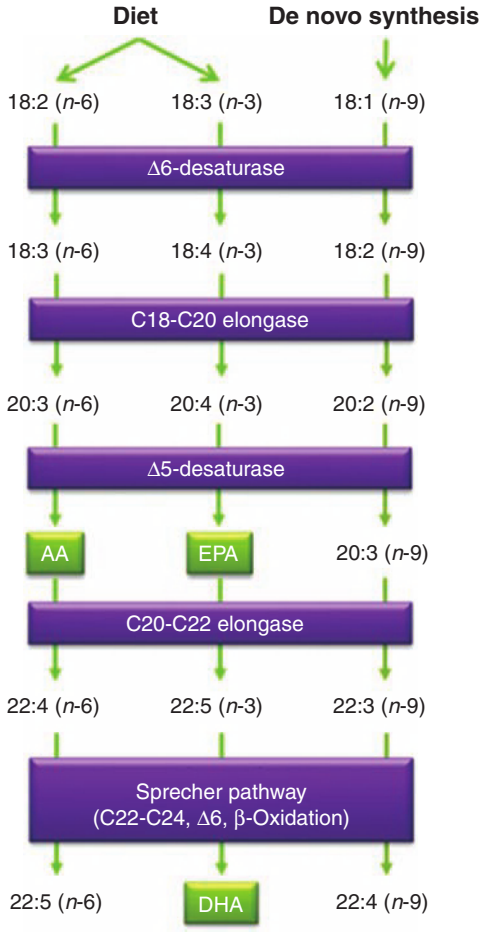

Plate 6.2 PUFA biosynthesis pathways. AA, arachidonic acid (20:4 *n*-6); EPA, eicosapentaenoic acid (20:5 *n*-3); DHA, docosahexaenoic acid (22:6 *n*-3).

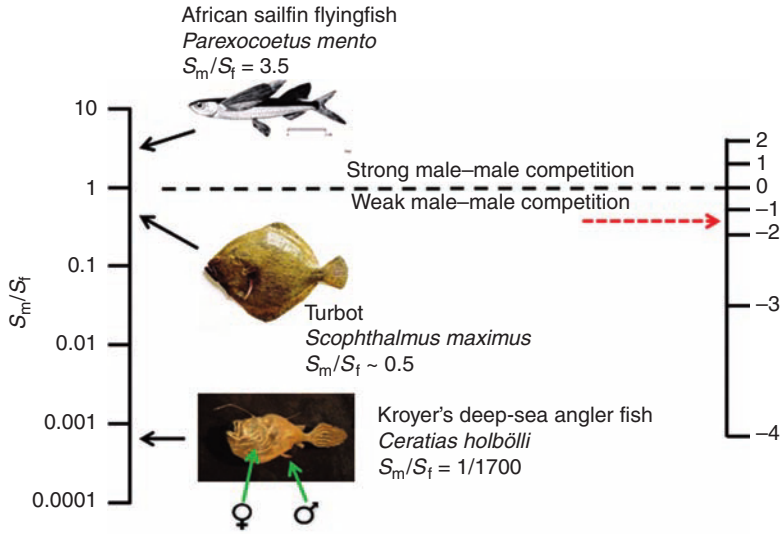

Plate 8.1 Sexual size dimorphism in fish. The vertical ratio scale on the left indicates the relation of the size (mass) of males, S_m, to that of females, S_f. In species with $S_m/S_f > 1$, males are larger than females, while in species with $S_m/S_f < 1$, the opposite is true. The ordinal scale on the right is based on the arbitrary classification of Parker (1992), with the following numerical meanings: 2, males larger than females; 1, males sometimes or slightly larger than females; 0, no size differences; −1, males somewhat smaller than females; −2, males smaller than females; −3, males much smaller than females but not dwarf; −4, true dwarf males. The black dashed line connecting the two scales indicates no size differences, whereas the red dotted arrows represents the mean value of -1.5 ± 0.2 obtained by Parker (1992) on the data of the 118 species present in Breder and Rosen (1966) for which information on sexual size dimorphism was available. Depicted are three examples, showing the extremes and the average situation. In the case of Kroyer's deep-sea angler fish–both sexes are shown (males are very small compared with females, live attached to them, and degenerate most organs but the gonads).

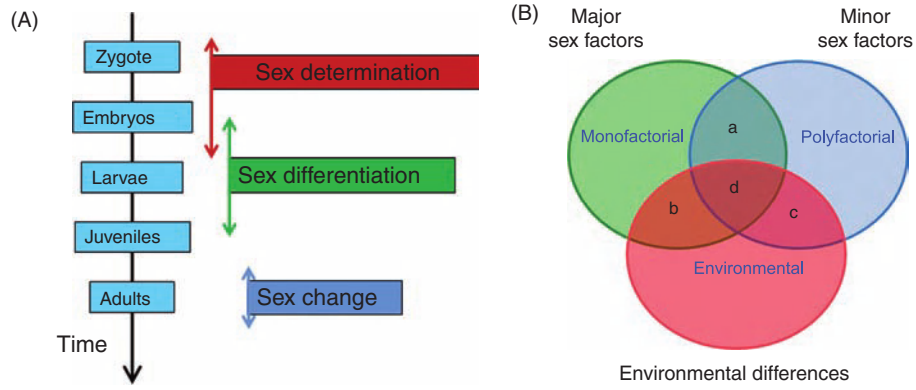

Plate 8.2 Sex determination and differentiation in fish. (A) The processes of sex determination, sex differentiation, and sex change are represented along the timeline of development. The arrows indicate the existence of interspecies differences in the timing of these processes. Sex change only occurs in sequential (also known as consecutive) hermaphrodites. (B) The three major components of fish sex determination. Genotypic sex determination (GSD) driven by major (green circle) and/or minor (blue circle) sex factors, and environmental sex determination (ESD), driven by environmental differences (red circle). Within each circle, the mechanism is indicated. Represented are species with: a, a combination of major and minor sex determining (SD) factors; b, major factors with environmental influences (rare); c, polyfactorial sex determination with environmental influences (common); and d, the theoretically possible combination of the three types of influences.

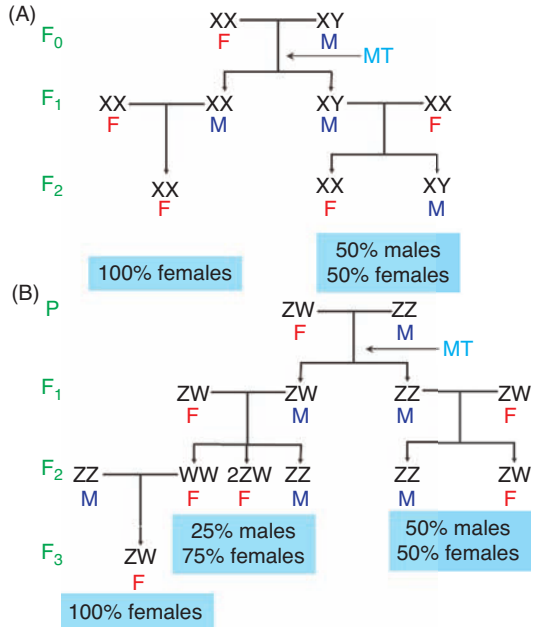

Plate 8.3 Endocrine methods to produce all-female stocks. Diagrams showing the indirect method of feminization by androgen treatment suitable for (A) species with female homogamety and (B) species with male homogamety, assuming the viability of the WW genotype. M, male; F, female; MT, 17α-methyltestosterone.

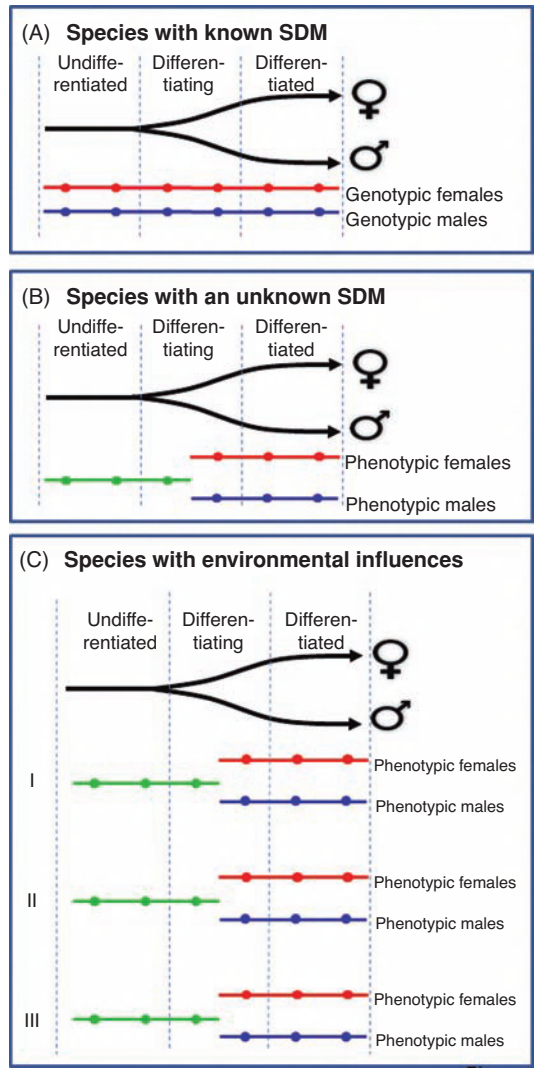

Plate 8.4 Strategies for the transcriptomic analysis of sex differentiation in fish. (A) species with known sex-determining mechanism (SDM); (B) species with an unknown SDM; and (C) species with environmental influences. RNA is harvested from genotypic females (red line), genotypic males (blue line), and undifferentiated fish (green line) at different times (dots) during development. In (C), I, II, and III indicate that the sampling has to be carried including fish reared under different environmental conditions.

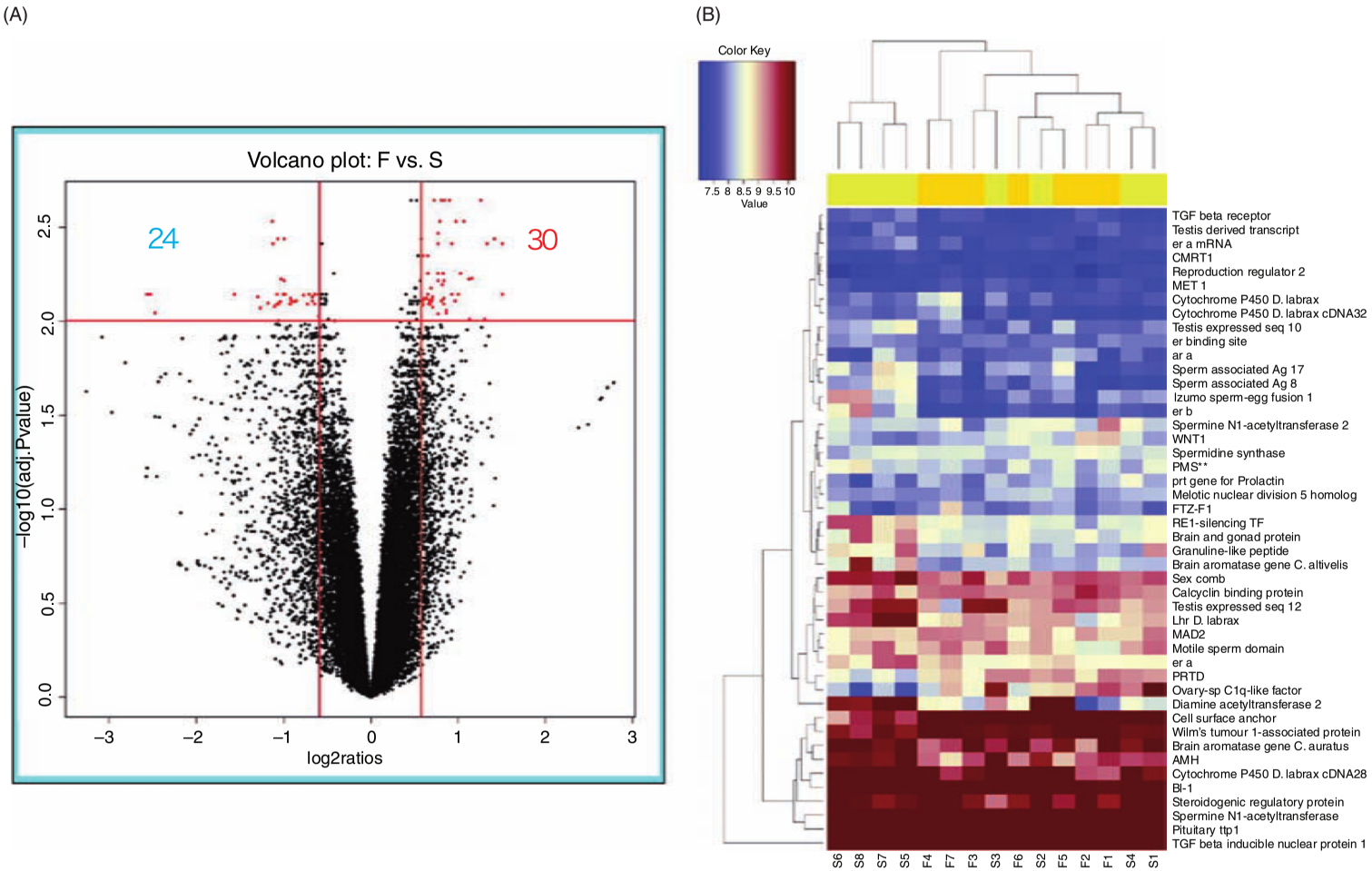

Plate 8.5 Transcriptomic analysis of sexually undifferentiated European sea bass sampled at 133 days postfertilization. (A) Volcano pot of genes being downregulated (blue) or upregulated (red) at a fold change > 1.5 with $p < 0.01$ between large (L) and small (S) European sea bass. (B) Heat map of reproduction-related genes between L and small S fish (Díaz et al., unpublished data).

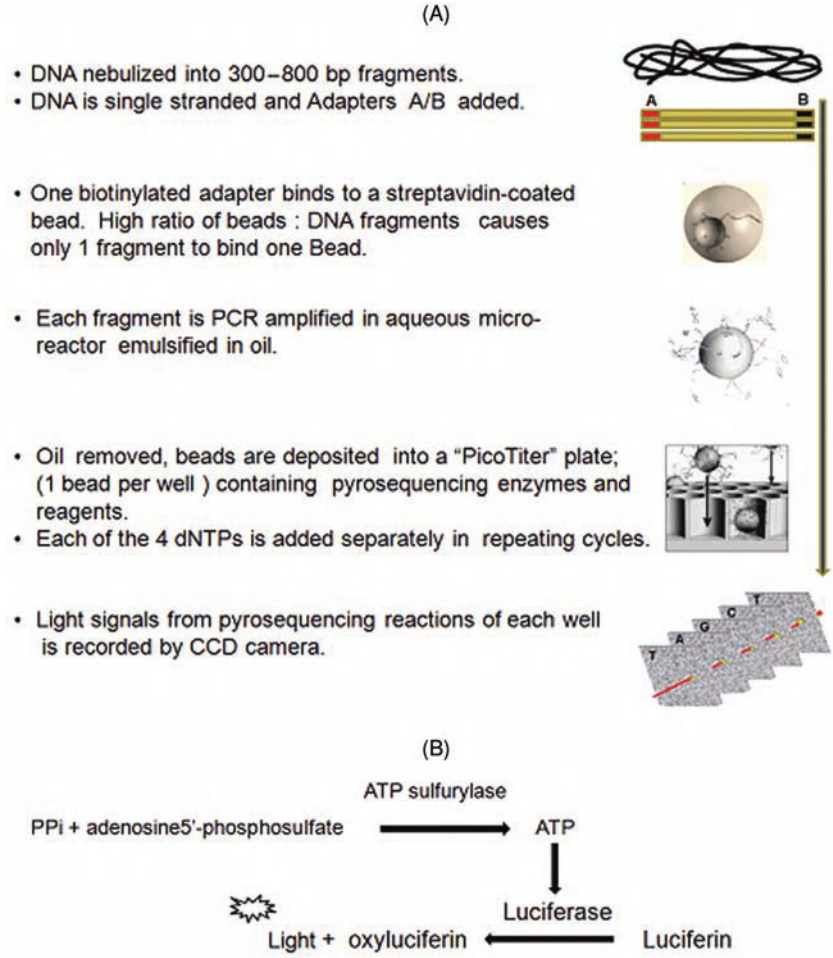

Plate 11.1 (A) Pyrosequencing workflow of the 454 Roche GS FLX and GS Junior platforms. (B) Pyrosequencing chemistry following addition of dNTPs to free 3′ end of the DNA fragment.

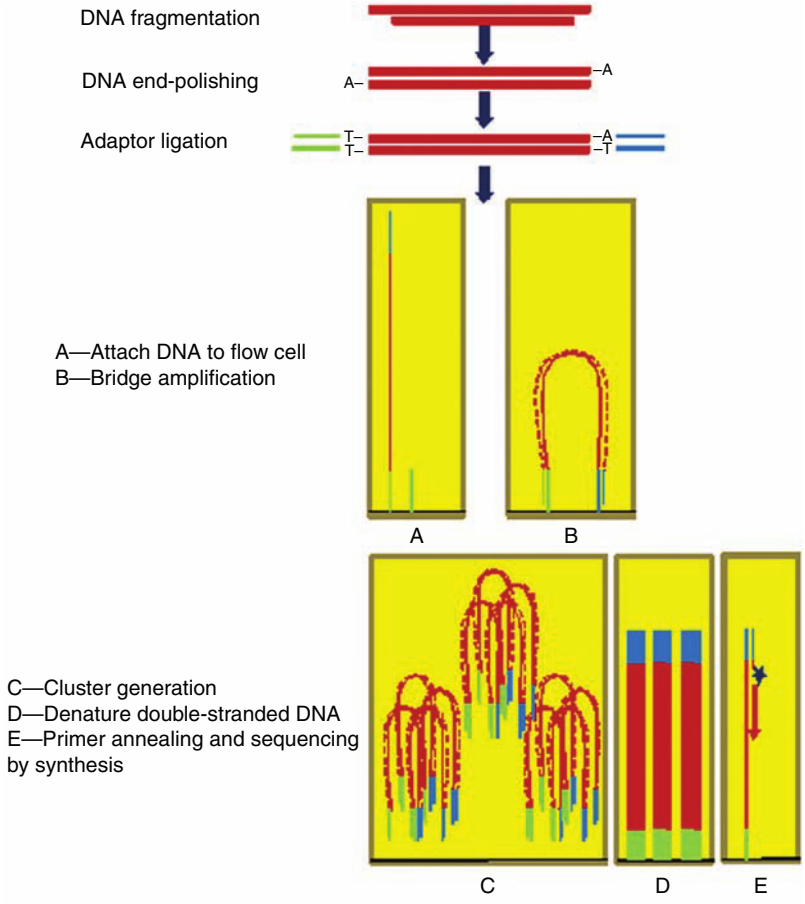

Plate 11.2 Illumina's sequencing-by-synthesis technology. Library preparation involves DNA fragmentation followed by end polishing and adaptor ligation. DNA fragments are immobilized onto flow cell and bridge amplified to form clusters. After cluster generation, the flow cell is loaded into the Genome Analyzer and subjected to repeated rounds of massively parallel sequencing-by-synthesis.

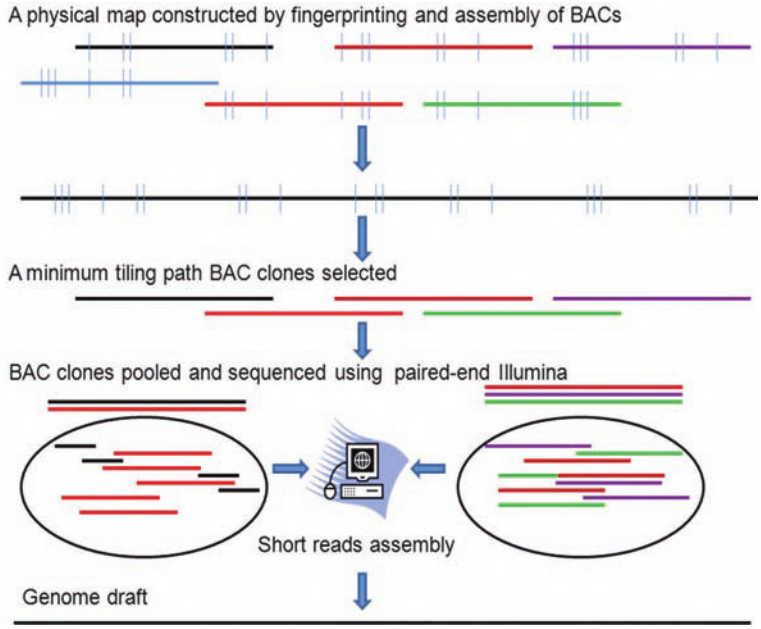

Plate 11.3 Application of NGS technology in sequencing the rainbow trout genome. BAC clones that constitute a minimal tilling path of the rainbow trout physical map are selected and pooled for sequencing using Illumina technology. Short reads from each pool are assembled into contigs. Contigs are aligned and joined together in a scaffolding process. A complete genome is drafted by positioning scaffolds along the physical map of the chromosomes.

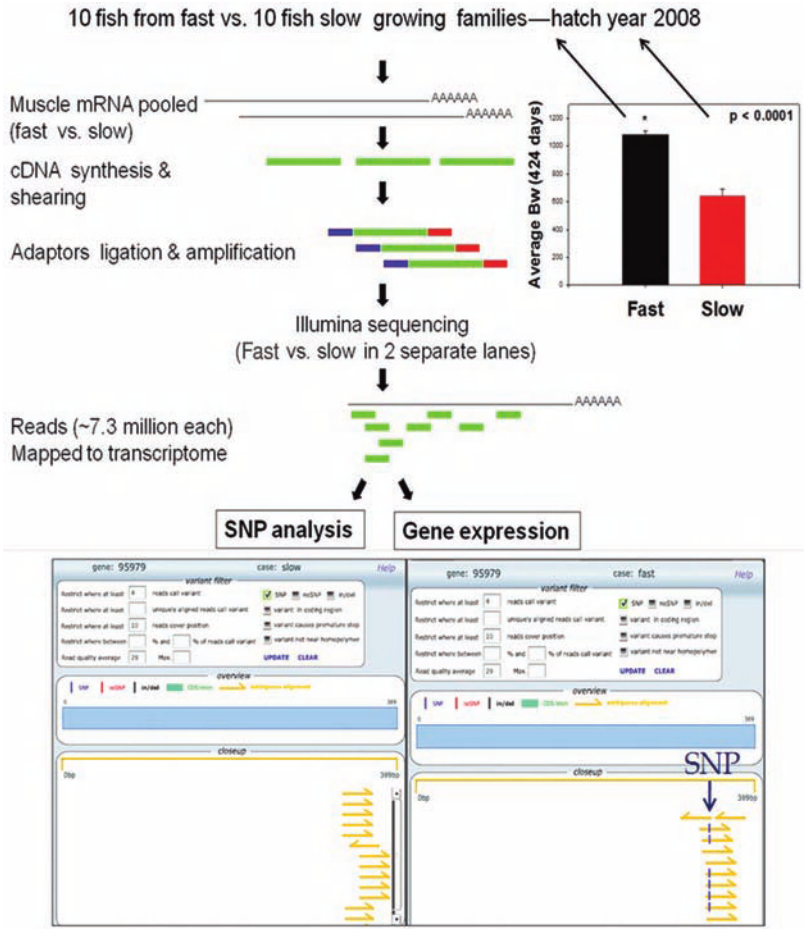

Plate 11.7 Upper panel, workflow of Whole Transcriptome Shotgun Sequencing (RNA-Seq) used to detecting genetic polymorphism in rainbow trout. Lower panel, an example of a type-I SNP marker identified in fast-growing (right) but not in slow-growing (left) rainbow trout fish population.

Chapter 1
Functional Genomics Research in Aquaculture: Principles and General Approaches

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Abstract: Functional analysis has always been more difficult, but it is nothing compared with structural analysis. This is especially true when the number of genes under study is increased to cover various systems and pathways on a genome scale. In this chapter, we provide an overview of functional genomics focusing on general approaches for functional genomics to include (1) Functional inference based on expression profiling such as analysis of expressed sequence tag (EST) analysis, microarray analysis, and RNA-Seq; (2) Functional inference of gene functions based on positional analysis such as genome-wide association studies (GWAS), quantitative trait loci (QTL) mapping, and expression quantitative trait loci (eQTL) mapping; (3) Functional inference of gene functions by comparative genome analysis; (4) Gene pathway analysis; and (5) Experimental determination of gene functions using novel technologies, such as the zinc finger nuclease (ZFN) technology. We also provide a section on epigenetics and analysis of protein–DNA interactions. At the end of the chapter, we offer our assessment of the potential of various technologies for functional genomics in aquaculture.

Introduction

Genomics as a branch of science started to make headway during the early to mid-1980s.

At the beginning, it started with a major research project "The Human Genome Project." As the genomic information was accumulated, and data was analyzed, a series of specific genomic methodologies were developed. With the rapid advances in technology, particularly the advances in PCR and sequencing technologies, a series of highly efficient approaches for genomic studies were developed. As a result of scientific demand and technological advances, a very specific branch of science evolved that is now called Genomics.

To gain better understanding of genomics, we must examine its roots. The term "genome" itself is more than 75 years old and refers to the entire genetic material of an organism, or its complete set of genes located on chromosomes (Hieter and Boguski, 1997). In 1986, "genomics" was coined by Thomas Roderick to describe the scientific discipline of mapping, sequencing, and analyzing genomes (Mckusick, 1989). The term of genomics has become universally accepted over the past two

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

decades. However, genomics is now undergoing a transition or expansion from the mapping and sequencing of genomes to an emphasis on genome functions. To reflect this shift, genome analysis may be generally divided into "structural genomics" and "functional genomics." Structural genomics represents an initial phase of genome analysis, studies the structure, organization, and evolution of genomes, while functional genomics, which studies expression and functions of the genomes. Structural genomics has a clear end point—the construction of high-resolution genetic, physical, and sequence maps of an organism. The ultimate map of an organism is its complete DNA sequence with a resolution of every single base pair (Hieter and Boguski, 1997). Although, genomics in its major research objectives can be divided into structural genomics and functional genomics, there is no clear separation of these subdisciplines. Furthermore, structural genomics is the basis for functional genomics.

Functional genomics represents a new phase of genome analysis (Hieter and Boguski, 1997). It requires the development of innovative technologies that make use of the vast resource of structural genomics information. Specifically, functional genomics refers to the development and application of genome-wide experimental approaches to assess gene functions by making use of the information and reagents provided by structural genomics. It is characterized by high throughput or large-scale experimental methodologies combined with statistical and computational analysis of the results. The fundamental strategy in a functional genomics approach is to expand the scope of biological investigation from studying single gene or protein to studying all genes or proteins at once on a genome-wide scale. Such operations allow the generation of tremendously large data sets that demand additional capacities of data analysis to draw information relevant to biology. Assistance is needed from all areas of biology, and more so from disciplines outside biology that can handle large data sets. Computer sciences and mathematics are among the first disciplines genomics has demanded cooperation from. Computational biology will play a critical and expanding role in this area: structural genomics has been characterized by data generation and management, whereas functional genomics will be characterized by mining the data sets for valuable biological information. Functional genomics promises to rapidly narrow the gap between sequence and function and to yield new insights into the behavior of biological systems (Hieter and Boguski, 1997).

The goal of this chapter is to provide some basic concepts of functional genomics, and provide a general description of approaches for functional genomics research in aquaculture.

The Concept of Functional Genomics

Genes and Gene Functions

Most eukaryotic organisms harbor tens of thousands of genes, and in most cases, perhaps more than 20,000 but fewer than 40,000 genes. Each gene has its own functions. Historically, gene functions were determined by observations of a phenotypic mutation followed by genetic mapping of the mutated phenotype and eventually trace to the gene controlling the trait. Such an approach is usually considered to be a forward genetics approach. This approach is highly straightforward; however, mutations, whether as a result of spontaneous mutation, or induced mutation, are rare and mutated phenotypes are oftentimes difficult to be observed in the first place. With the rapid progress of DNA sequencing technologies, scientists started to know much quicker about a specific gene sequence and its protein structures than the functions of the gene. As a result, a new set of reverse genetics approaches was developed. In reverse genetics, the simple concept is to inactivate the gene, and then determine the changes of the phenotypes; alternatively, to add more of the gene products, and then determine the

changes of the phenotypes. In the former, genes can be specifically targeted to "knockout" the gene. This worked really well with some model species where embryonic stem cell technology is available. For instance, with rat or mouse, a specific gene can be knocked out in an embryonic cell line, and then individuals can be developed from the cells, and phenotypes can be observed in the "knocked out" animals. However, this approach is to date not applicable to aquaculture species because embryonic stem cell technologies have not been developed for aquaculture species.

In recent years, the discovery of RNA interference (RNAi) has lent research tools for the study of gene functions. RNAi is an RNA-dependent gene silencing process that has been applied to knockdown gene expression (Hannon, 2002). This is particularly useful for some aquaculture species, as RNAi technologies have been applied to investigate gene function as well as to develop antiviral agents to combat various infections in some fish and crustaceans species (Acosta et al., 2005; Copf et al., 2006; Liu et al., 2006; Wargelius et al., 1999; Kelly and Hurlstone, 2011). For instance, the highly efficient gene knockdown was observed to result in similar embryonic defects to the known mutant phenotypes in zebrafish (Wargelius et al., 1999). In a study of genes expressed differentially in freshwater crayfish infected with the white spot syndrome virus (WSSV), the anti-lipopolysaccharide factor gene (AF) was shown to protect against WSSV infection, and knockdown of AF by RNAi specifically resulted in higher rates of viral propagation (Liu et al., 2006).

In addition to gene knockout and knockdown technologies, transgenic technology has been widely used to demonstrate the functions of genes. The basic principle is that if a gene has certain functions, its over-expression should cause changes in phenotypes. Such an approach was best demonstrated by transgenic fish harboring the growth hormone gene that grow much faster and bigger than their non-transgenic controls (Du et al., 1992; Gross et al., 1992; Devlin et al., 1994; Rahman et al., 1998; Rahman et al., 2001).

All the traditional approaches for the study of gene functions are effective, but they have fatal problems that include the following:

1. Not all genes can cause a visible phenotype.
2. Knockout of one gene may cause numerous other changes in genome expression; many of these are compensatory or consequential, making analysis of gene functions very difficult.
3. Functional study of gene function by "one gene at a time" is too laborious, too expensive, and too slow.

As a result, scientists have explored to study gene functions on the genomic scale that leads to the emergence of functional genomics.

Concept of Functional Genomics

Functional genomics can be defined as a discipline for the understanding of gene functions and regulation on a genome-wide scale. It is a field of molecular biology that attempts to make use of the vast wealth of data produced by genomic projects (such as genome sequencing projects) to describe gene (and protein) functions and their interactions. Unlike structural genomics and proteomics, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein–protein interactions and interactions between proteins, DNA, and RNA. As opposed to the static aspects of the genomic information such as nucleotide and amino acid sequences or structures, functional genomics attempts to answer questions about the functions of DNA at the levels of genes, RNA transcripts, and protein products (Hackett and Clark, 2007). The ultimate goal for functional genomics is to bridge the gap between the blueprint (genome sequence or genotype) and the living organism (trait or phenotype) under various environmental conditions (Cogburn et al., 2007). Spawned by the technological revolution in genome

sequencing (Venter et al., 1996; Rowen et al., 1997), functional genomics studies currently are greatly stimulated by the high-throughput sequencing and screening technologies. Therefore, a key characteristic of functional genomics studies is the genome-wide approach generally involving high-throughput technologies rather than the traditional "gene-by-gene" approach.

Goals of Functional Genomics

The goals of functional genomics are to gain better understanding of the roles of functional elements in the genome that directly or indirectly affect the development, growth, metabolism, immunity, behavior, reproduction, and various other processes of an organism. One of the primary tasks of functional genomics is assigning specific functions to genes, noncoding RNAs, and *cis*-Acting DNA elements involved in the processes explained earlier (Hackett and Clark, 2007). The goals of functional genomics vary depending on organism and project. In the broad sense, functional genomics studies are conducted with the following objectives:

1. To discover functional elements from genomic sequence of organisms, including protein coding genes and regulatory noncoding regions;
2. To obtain the global assessment of how the expression of all genes in the genome varies under changing conditions;
3. To generate resources and develop methodologies for genome-wide mutagenesis deducing the functions of novel genes by mutating them and studying the mutant phenotype;
4. To genetically manipulate organisms for specific purposes;
5. To understand the evolution of genomes in relation to biology of organisms across a spectrum of evolutionarily-related species.

Approaches to Functional Genomics

Numerous approaches with sophisticated experimental techniques have been developed for functional genomics studies. The huge numbers of genes, and even larger numbers of transcripts contribute to the complexity of functional genomics analysis. Such complexities are amplified again by an even greater diversity of polypeptides and their post-translational modifications, the variation in expression from different tissues under different developmental stages, and under various environmental conditions (Hackett and Clark, 2007). High throughput methodologies are required to simultaneously examine the expression and functions of all genes of an organism. Clearly, high-speed computation is an essential feature of most functional genomics techniques. In the later sections, we describe some of the general approaches currently used in functional genomics studies, with the understanding that more powerful methods are constantly emerging, especially as computational power continues to improve. The improvements include tools and instruments for digital recording of information and newer algorithms for sorting and analyzing information developed as our understanding of gene function increases. Because of the large quantity of data generated by these techniques and the desire to discover biologically meaningful patterns, bioinformatics is crucial to functional genomics data analysis (Hackett and Clark, 2007).

While whole genome sequencing is now a routine, functional analysis continues to be a great challenge. There are several general strategies for elucidating the functions and regulation of gene expression. In terms of strategies, functional genomics approaches can be divided into four general categories: (1) Functional correlation of gene expression and thereby inference of gene functions; (2) Functional correlation of gene positions and thereby inference of candidate gene functions; (3) Functional assignment by association

with gene pathways; and (4) Direct testing of gene functions.

Functional Correlation of Gene Expression Profiling

Short of direct testing that is often very difficult, gene functions can be inferred from their correlations of expression with function, or their correlations of position with function. The basis for functional inference from expression correlation is expression profiling. The idea is that if a gene is involved in a specific trait, e.g., defense response, its expression would respond to infection. Gene expression profiling is the measurement of expression levels for thousands of genes to paint a general global picture of gene expression under a specific developmental stage, environmental condition, or treatment. In practice, gene expression profiling experiments often involve measuring the relative amount of mRNA expressed in two or more experimental conditions ("treatment"). The correlation of "treatment" and gene expression profile could provide inference on gene functions.

Various technologies have been developed to quantify gene expression, including hybridization-based and sequence-based approaches. Hybridization-based approaches typically involve incubating fluorescently-labeled complementary DNA (cDNA) with custom-made microarrays or commercial high-density oligo microarrays. In contrast to microarray methods, sequence-based approaches determine gene expression levels by directly sequencing cDNAs. The relative abundance of cDNAs reflect gene expression levels. Initially, Sanger sequencing of cDNA or expressed sequence tag (EST) libraries were used (Gerhard et al., 2004), and then tag-based methods were developed to improve the throughput, including serial analysis of gene expression (SAGE) (Velculescu et al., 1995; Harbers and Carninci, 2005) and massively parallel signature sequencing (MPSS) (Brenner et al., 2000). These tag-based sequencing approaches are high throughput and can provide precise, digital gene expression (DGE) levels. Recently, the development of high-throughput DNA sequencing technologies provides additional strengths for gene expression profiling. This method, termed as RNA-Seq, has clear advantages over previous sequence-based approaches and is expected to revolutionize the manner in which transcriptomes are analyzed for both gene discovery and global gene expression profiling.

In terms of functional genomics, the largest portion of published literature to date involves gene expression profiling through sequencing-based approaches such as EST analysis, SAGE, MPSS, and more recently developed RNA-Seq, and hybridization-based microarray analysis. We will limit our discussion to EST analysis, microarrays, and RNA-Seq as these are the most commonly used approaches.

Analysis of Expressed Sequence Tags (ESTs)

ESTs are single-pass sequences of random cDNA clones from cDNA libraries. They are traditionally generated using Sanger sequencing and therefore the resultant sequences are approximately 500 to 800 base pairs in length. Several years ago, because sequencing was relatively cheap, large numbers of ESTs can be generated at a reasonably low cost from either the 5′ or 3′ end of a cDNA clone to get an insight into transcriptionally active regions. ESTs were used as a primary resource for human gene discovery (Adams et al., 1991). Thereafter, there has been an exponential growth in the generation and accumulation of EST data in public databases for various organisms, with approximately 71 million ESTs now available in public databases (http://www.ncbi.nlm.nih.gov/dbEST/, September 2011, all species). Readers can refer to Chapter 2 of this volume for availability of ESTs among various aquaculture species.

EST analysis is an effective genomic approach for rapid identification of expressed genes, and has been widely used in genome-wide gene expression studies in various tissues, developmental stages or under different environmental conditions (Adams et al., 1995; Ronning et al., 2003). In addition, the availability of cDNA sequences has accelerated further molecular characterization of genes of interest and provided sequence information for microarray construction and genome annotation (Bailey et al., 1998; Lo et al., 2003; Kim et al., 2006).

Gene expression analysis plays an important role in identifying differentially expressed genes under different environmental conditions and gene expression regulation, shedding light on gene functions. EST analysis has been demonstrated effective for detection of differential expression and regulation of certain genes. Without normalization or subtraction in library construction, the number of the sequenced ESTs for a given gene reflected the abundance of the gene expression at the corresponding scenario (e.g., environmental conditions, developmental stages, treatments, etc.).

Direct EST sequencing is inefficient in discovery of rarely expressed genes. To solve this problem, the method to construct normalized cDNA libraries was developed (Soares et al., 1994; Bonaldo et al., 1996). The basic principle is using hybridization to reduce redundant genes and increase the representation of rarely expressed genes.

Initial annotation of ESTs can be conducted by simple sequence similarity comparisons. Further annotation analysis can be carried out after obtaining the consensus sequences (putative unigenes), such as determination of gene identity based on homology search, open reading frame (ORF) identification, Gene Ontology (GO) annotation and gene-enrichment analysis (e.g., Nakaya et al., 2007).

In order to assign gene identity to contigs and singletons, homology search is widely used. Such an approach is especially helpful for newly-studied species. BLAST is the most widely used program to obtain high throughput EST analysis and annotation results. BLAST package provides different flavors of algorithm for sequence similarity searching. BLASTX is used to search against protein database by translated consensus EST sequences while BLASTN is used to search against nucleotide sequence databases. NCBI, ENSEMBL and Swiss-Prot are three important databases for BLAST search. For instance, Swiss-Prot database have fully manually curated and annotated unigene database, Uniprot, which can be used for identifying putative function for unigene by BALSTX. NCBI provide dbEST database that can be used to search novel transcript by BLASTN. dbEST is a main ESTs resource database including ESTs for over 200 aquaculture species. ENSEMBL database can provide chromosome location information of genes, which is a useful tool for comparative genome analysis. However, BLAST sequence similarity comparison provides only sequence homology information, and one cannot purely rely on BLAST for gene identification. Detailed phylogenetic analysis and/or orthology analysis is needed to determine the identities of genes.

For a greater level of annotation, ORF is identified to determine the full or portion of coding region in the unigene. The unigene with a full ORF usually represent a full-length cDNA. There are some useful tools for ORF detection. For example, ESTScan (Iseli et al., 1999) can extract coding regions from low-quality ESTs and correct frame shift errors. OrfPredictor (Min et al., 2005) is another program for identification of protein-coding sequences from ESTs through predicting most probable coding regions from all the six translation frames.

GO annotation can provide description of gene products behaving in a cellular context. Gene functions are placed into three categories: biological processes, cellular components, and molecular functions. Consensus sequences can be linked to GO terms

and assigned a possible function by Blast2GO (Conesa et al., 2005).

GO enrichment analysis is to cluster most relevant GO terms associated with certain biological pathway. GOEAST (Zheng and Wang, 2008), Ontologizer (Bauer et al., 2008), GeneTrail (Backes et al., 2007), and DAVID functional annotation tool (Huang et al., 2009) are useful tools for these analysis.

EST analysis is an efficient approach for gene discovery and gene identification. For instance, during 2001 to 2007, catfish ESTs increased from 10,000 to 44,000 and the putative genes number increased from 5905 to 25,000 (Li et al., 2007). In Pacific oyster (*Crassostrea gigas*), 40,845 high-quality ESTs represented 29,745 unique transcribed sequences (Fleury et al., 2009). In gilthead sea bream (*Sparus auratus*), 30,000 ESTs represented 18,196 putative unigenes (Louro et al., 2010). Currently, there are over 180 aquaculture species having more than 100 ESTs in dbEST (see Chapter 2 of this volume on existing genome resources for details).

EST analysis can provide comparisons of gene expression profiling in different tissues and conditions. For instance, in a recent study with rainbow trout (*Oncorhynchus mykiss*), Kondo et al. (2011) sequenced over 30,000 ESTs from rainbow trout adipose tissue. These ESTs were used to search adipokine-related genes. The result showed that none of them encoded adipokine and PPAR-γ gene, which play important roles in mammalian adipocytes. Further qRT-PCR result confirmed EST analysis results, that is, rainbow trout adiponectin transcripts were weakly detected in adipose tissue but strongly detected in muscle, suggesting the difference of energy metabolism between fish and mammal (Kondo et al., 2011). Chini et al. (2008), constructed normalized cDNA libraries from liver, ovary and testis in blue fin tuna (*Thunnus thynnus*), identifying several sequences with known function in other organisms, but not previously described in this species. Also, sequences were described being expressed in one, two, or more tissue libraries. Similarly, Zou et al. (2011) constructed normalized cDNA libraries from testis, ovary, and mixed organs of mud crab (*Scylla paramamosain*). Through EST analysis, sex-specific transcripts were identified.

EST resources provide sequence information for microarray development. For instance, in a recent study, Booman et al. (2011) developed a large-scale oligonucleotide microarray platform containing 20,000 features (20K), which was used to study immune response of the Atlantic cod spleen with stimulation of formalin-killed, atypical *Aeromonas salmonicida* (Booman et al., 2011). Similarly, oligo microarray for gilthead sea bream (*Sparus aurata*) was developed based on ESTs, and the microarray was used to identify 1050 differentially expressed genes between two developmental stages (Ferraresso et al., 2008).

Although EST analysis has been important for transcriptome characterization, it is now becoming expensive, relative to several of the most recently developed approaches, as described in the following text. However, EST resources still have a great value to serve as reference for RNA-Seq analysis. We found that ESTs are essential for high-quality reference-guided assembly of next-generation sequencer-generated short reads (Liu et al., 2011).

Microarrays

Microarray is a powerful tool that allows analysis of global gene expression in individual cells or tissues under different conditions (Schena et al., 1995). The core principle of microarray is dense placement of gene target sequences in a small area and hybridization. Tens of thousands of DNA sequences termed probes are anchored or spotted onto the solid surface of a chip. Fluorescence-labeled probes are used to hybridize with the features on the microarray. Microarray combines simple nucleic acid hybridization with high-density spotting robots, fluorescence-based signal detection and high-resolution laser scanners (Peatman and Liu, 2007). High-density spotting

robots and photolithography allow each feature to be placed accurately on the slide in high densities. Fluorescence-labeled probe provides much clearer signal than the traditional radiation labeling. Moreover, the high-resolution laser scanner allows accurate fluorescence-signal quantification.

Based on the construction and sample labeling, there are two primary approaches to DNA microarray used in aquaculture species: the spotted arrays (or printed arrays) and the in situ arrays. Spotted arrays are constructed by spotting cDNA, small fragments of PCR products or long oligos using robot. This technique is adapted by most researchers to produce "in-house" printed microarray, because it is relatively low-cost and flexible. The researchers can decide the probes, generate their own probes, spot the array, hybridize the samples to the array, and scan the arrays with their own machine. However, it is labor-consuming. The number of spots (features) is limited to avoid cross-contamination. Two-color fluorescence, such as Cy3 and Cy5, are usually used for sample labeling for spotted array (Schena et al., 1996).

In situ arrays are constructed by synthesizing short oligos directly onto the slide surface by photolithography instead of depositing intact sequences. The oligo probes may be longer, like 65-mer (Mathavan et al., 2005), or shorter, like 24-mer (Peatman et al., 2007). Longer probes are more specific, whereas the shorter ones are cheaper and can be spotted in higher densities. In order to overcome the short probes to improve the specificity and sensitivity, in situ arrays contain high-density features, usually multiple probes per target (Miller and Tang, 2009). A perfect match (PM) and mismatch (MM) system is used to further improve the specificity (Irizarry et al., 2003; Han et al., 2004). MM probes contain one or more mismatched nucleotides within the PM probe sequences and act as a negative control to detect the false-positive signal resulted from the nonspecific cross hybridization.

Affymetrix (http://www.affymetrix.com/) is one of the most widely known industries for in situ array. Semiconductor-based photochemical synthesis and photolithographic masks are used to synthesize oligo probes for Affymetrix GeneChip. The photolithographic masks either block or allow light to reach the microarray surface. In the area the mask covers, the addition of the nucleotides will be prevented since the UV light has been blocked, whereas in the area exposed to the UV light, the specific nucleotide can be added. After many cycles of unmasked, addition of nucleotide and masked, the sequences of every oligo probes are fully constructed. Another commercial microarray manufacturer Roche NimbleGen (http://www.nimblegen.com/) has developed a maskless array synthesis, which uses digital mirrors instead of the photolithographic masks (Nuwaysir et al., 2002) at a significantly low start-up cost. Agilent Technologies construct the oligo probes for in situ array use glass slides and inkjet printing, neither photolithographic masks nor digital mirrors. Instead of Cy3/Cy5 labeling system, the sample using in situ array is usually Biotin–Streptavidin labeled. No matter which platform is used, spotted array or in situ array, the basic procedure for gene expression experiment is similar, starting with RNA. As shown in Figure 1.1, the RNA is extracted from the sample that we are interested and reverse transcribed to cDNA after quantification and quality check. The cDNA is fluorescently labeled and hybridized to the probes on the microarray. The hybridization will result in fluorescence signal, which can be measured by a fluorescence scanner and then be analyzed by using software, e.g., R/Bioconductor (http://www.bioconductor.org). Background correction and data normalization are conducted then to minimize variation caused by nonbiological effects (Xiang and Chen, 2000), followed by cluster analysis (Eisen et al., 1998), which establish gene expression patterns and define the relationships between gene expression profiles across different samples (e.g., treatment sample vs. control sample).

When starting the microarray experiment, researchers need to keep in mind that all

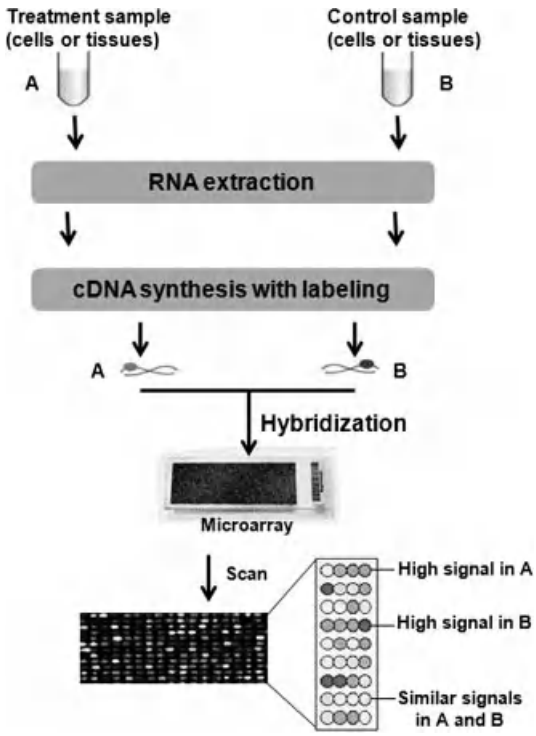

Figure 1.1 A schematic presentation of microarray experiment where expression of two samples are compared with A being the sample under treatment, and B being the sample for control. RNA is separately isolated from the samples, and fluorescently-labeled probes are made separately with different labels, e.g., cy3 for treatment (green), and cy5 for control (red). The probes are simultaneously used to hybridize an array containing the features representing the transcripts of the organism. After hybridization, signals are scanned and analyzed. If the signals are high in the treatment, green will be detected, and if the signals are high in control, red will be detected; if the signals are equal, yellow will be detected. Based on the relative signals of red and green, expression levels are determined. See color insert.

designs should meet the standards of the Microarray Gene Expression Data Society (MGED) and be compliant with the Minimum Information About a Microarray Experiment (MIAME) guidelines (http://www.mged.org/Workgroups/MIAME/miame.html). Depending on biological questions they are interested and availability of the financial and genetic resources, the researchers need to make appropriate decisions to construct the microarray experiment.

In recent years, along with more and more genetic resources available, such as ESTs, transcriptome sequences, whole genome sequences and so on, microarray technologies have been dramatically advanced and broadly applied. A variety of microarrays including low-density or high-density cDNA arrays and oligo arrays have been developed in aquaculture species, such as zebrafish (Ton et al., 2002; Mathavan et al., 2005), Salmonidae (von Schalburg et al., 2005; Koop et al., 2008), catfish (Li and Waldbieser, 2006; Peatman et al., 2007), Atalantic cod (Booman et al., 2011; Edvardsen et al., 2011), shrimp (Wongsurawat et al., 2010; Aoki et al., 2011; Leelatanawit et al., 2011), oyster (Wang et al., 2010; Dheilly et al., 2011), and so on. See Chapter 2 for information on the detail microarray resources. The applications of microarray in aquaculture species are mainly focused on the following aspects: (1) *Development*: Determining how genes interact and change the expression level during developmental process has been the main goal for development biology. Microarray, an efficient technology to globally identify the patterns of gene expression, is well utilized in aquaculture for development especially embryogenesis. Ton et al. (2002) constructed a zebrafish cDNA array to reveal dynamic change in levels of gene expression involved in development (Ton et al., 2002). An Atlantic cod cDNA microarray representing 7000 genes were used to analyze the temporal activity of the transcriptome during early cod embryogenesis (Drivenes et al., 2011). (2) *Immunity or disease resistance*: Many studies have been done by using microarray to screen and identifying genes that are involved in the immune system and disease resistance. For instance, Meijer et al. (2005) use a zebrafish oligo array containing 16K features to analyze a host transcriptome response to mycobacterium *Mycobacterium marinum* infection at the

organismal level (Meijer et al., 2005). 28K oligo arrays have been developed in catfish to identify immune-related genes in catfish (Peatman et al., 2007). (3) *Response to environmental variation or stress*: Environmental variations, such as hypoxia, water temperature, and salinity, will cause changes in physiology, genomics, and gene expression for aquaculture species. A microarray containing 8046 medaka unigenes was developed to measure gene expression profiling in the brain, gill, and liver of medaka after exposed to hypoxia (Ju et al., 2007). Microarray analyzes also have been conducted on gene expression change in water temperature (Kassahn et al., 2007; Hirayama et al., 2008). (4) *Reproduction*: Reproduction is an important trait in aquaculture industry. Karoonuthaisiri et al. (2009) utilized a cDNA microarray to screen reproduction-related genes in giant tiger shrimp, and several transcripts were identified that play important roles during shrimp ovarian development.

Currently, microarrays are mainly used to accelerate gene expression analysis under various experimental conditions. In the future, it looks promising to use microarray for single nucleotide polymorphisms (SNP) analysis, quantitative trait loci (QTL) mapping, and disease diagnosis. However, microarray study in aquaculture species is in its infancy, mostly because of the incomplete whole genome sequences in most aquaculture species. It is an essential task for the aquaculture community to exploit and adapt the advances for their respective species.

High Throughput Sequencing of mRNA (RNA-Seq)

RNA-Seq takes advantage of high-throughput DNA sequencing technology to capture the complete set of mRNA transcripts in a cell of an organism (Nagalakshmi et al., 2010). In this approach, mRNA is reverse transcribed into cDNA and fragmented, then sequenced using a next-generation technology to generate reads that can be assembled to cover a good portion of the transcripts, if not the full length of transcripts (see illustrations in Figure 1.2). Based upon different choices of sequencing technology, the sequencing yields and read lengths vary.

Currently, three main next-generation sequencing platforms are widely used in the RNA-Seq, the 454, Illumina and ABI SOLiD. Among these platforms, the throughput varies from hundreds of thousands of reads for the 454 system to hundreds of millions of reads for the Illumina and ABI SOLiD systems (Marguerat and Bahler, 2010). The read lengths typically range from 30–100 bp for Illumina and SOLiD to 200–500 bp for 454. In general, Illumina and SOLiD platforms are relatively inexpensive, while the 454 technology offers longer reads, but is more expensive per run. Illumina, SOLiD and 454 technologies can be combined in a "hybrid assembly" strategy: short reads that are sequenced at a greater depth are assembled into contigs, and long reads are subsequently used to scaffold the contigs and resolve variants (Martin and Wang, 2011).

Two main approaches can be used for RNA-Seq data analysis. One way is to map the resulting reads to a reference genome or reference transcriptome. This is usually taken in well-studied species with sequenced genome. The other way is to do the de novo assembly for species without reference genome or transcriptome. Consequently, a genome-scale map that is composed of both the transcriptional structure and/or level of expression for each gene can be generated (Wang et al., 2009).

RNA-Seq, as a way of high-throughput sequencing method, is being widely used in functional genomics studies in aquaculture species and their related model fish species such as zebrafish (Hegedus et al., 2009; Aanes et al., 2011; Bontems et al., 2011; Ordas et al., 2011; Rosel et al., 2011; Vesterlund et al., 2011), catfish (Liu et al., 2011), Japanese sea bass (Xiang et al., 2010), Atlantic cod (Johansen et al., 2011), large yellow croaker (Mu et al., 2010), rainbow trout (Lewis et al., 2010; Salem

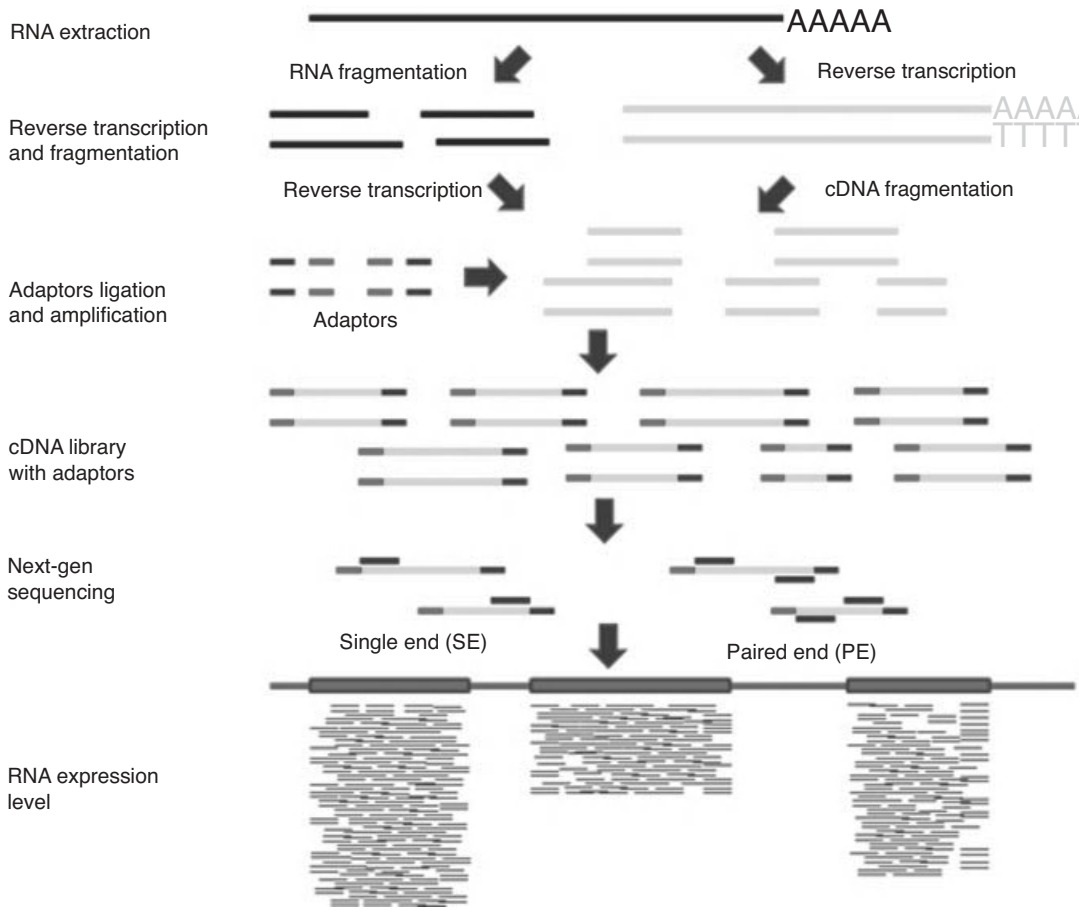

Figure 1.2 A schematic presentation of RNA-Seq. The extracted RNA is first converted into a library of complementary DNA (cDNA fragments through either RNA fragmentation (left) or DNA fragmentation (right). Sequencing adaptors (depicted by short red bars and short purple bars) are subsequently ligated to each cDNA fragment (green lines) and short sequence reads (single end or paired ends) from each cDNA are generated using high-throughput sequencing technology. The resulting sequence reads [short lines beneath the genome sequence with three genes shown (fat blue bars)] are aligned with the reference genome to evaluate gene expression by counting mapped reads. In the given example, the gene on the very left are expressed at a very high level, and the gene in the middle is expressed at a relatively lower level. See color insert.

et al., 2010; Purcell et al., 2011), European eel (Coppe et al., 2010), and spotted gar (Amores et al., 2011). The applications of RNA-Seq in aquaculture species are focused on these aspects: (1) Gene expression profiling, (2) Transcriptome characterization and gene annotation, and (3) Identification of gene-associated markers.

RNA-Seq can be used to identify differentially expressed genes under different treatments by measuring the expression level. For instance, in the study of transcriptome changes in zebrafish with mycobacterium infection (Hegedus et al., 2009) and zebrafish embryos with Salmonella infection (Ordas et al., 2011), Illumina's DGE system revealed the

high degree of transcriptional complexity of the host response to both infections and resulted in the discovery of a common set of infection-responsive genes with induced expression in infected individuals. Stockhammer et al. (2010) used the combination of microarray analysis and whole transcriptome deep sequencing to analyze the response to bacterial infection with emphasis on identification of a gene set whose responsiveness during infection is highly dependent on Traf6. In a study with large yellow croaker infected with *Aeromonas hydrophila*, changes of multiple signaling pathways involved in immunity were revealed, which will facilitate the comprehensive understanding of the mechanisms involved in the immune response to bacterial infection (Mu et al., 2010). Deep sequencing-based transcriptome profiling analysis of bacteria-challenged Japanese sea bass provided insight into the immune-relevant genes in marine fish. In the study, over 1000 strong infection-responsive transcripts were identified as significantly up- or down-regulated genes, suggesting the considerable alteration of the host transcriptome profile after the *Vibrio harveyi* infection (Xiang et al., 2010). In a study focusing on gene expression changes during development, RNA-Seq is used to compare the transcription profiles of four early developmental stages in zebrafish on a global scale. An enrichment of gene transcripts with molecular functions of DNA binding, protein folding and processing as well as metal ion binding was observed with progression of development (Vesterlund et al., 2011).

Transcriptome characterization and gene annotation is another area RNA-Seq can be applied. Transcriptome data generated through RNA-Seq can provide accurate and effective reagents for annotating the protein-coding genes. Despite the availability of complete genome sequences, a complete genome annotation would require knowledge of all transcription start and polyadenylation sites, exon–intron boundaries, splice variants, and regulatory sequences (Morozova et al., 2009). Because of its longer read lengths compared with other new sequencing technologies, 454 has been effectively used for de novo assembly of the transcriptome in several aquaculture species including lake sturgeon (Hale et al., 2009), rainbow trout (Salem et al., 2010), Atlantic cod (Johansen et al., 2011), and Yesso scallop (Hou et al., 2011). In an early study to characterize gene expression of gonad transcriptome in polyploid lake sturgeon using 454 sequencing, thousands of contigs were assembled and characterized from 454 reads providing an overview of transcription in lake sturgeon gonads, including the discovery of the genes and SNPs (Hale et al., 2009). High-throughput sequencing of the rainbow trout transcriptome using 454 sequencing technology significantly increased the suite of ESTs available for rainbow trout, allowing improved assembly and annotation of the transcriptome (Salem et al., 2010). A more recent study in the guppy, de novo assembly of the guppy (*Poecilia reticulata*) transcriptome using 454 sequence reads were conducted to detect sex-specific transcripts and provide a reference for gene expression analysis (Fraser et al., 2011).

Although shorter reads produced by Illumina or SOLiD compared with the 454 technology may be more challenging for de novo sequence assembly, the preexisting ESTs produced by Sanger sequencing can be used to facilitate the assembly (Liu et al., 2011), and the algorithms for short reads de novo assembly are being developed (e.g., Grabherr et al., 2011). Xiang et al. assembled the short reads from Illumina RNA-Seq deep sequencing to generate the nonredundant consensus which is subsequently used as references for DGE profile analysis (Xiang et al., 2010). RNA deep sequencing of the Atlantic cod transcriptome was conducted using the combination of 454, Illumina and ABI SOLiD platforms to increase the efficiency of assembly (Johansen et al., 2011).

RNA-Seq has been extensively used for the identification of gene-associated markers. In catfish, hundreds of thousands of gene-associated SNPs have been identified by deep

sequencing of RNA from many individuals of both channel catfish and blue catfish, which will be used in development of high-density catfish SNP chips for genome-wide association studies (GWAS) (Liu et al., 2011). In the study to understand the adaptive divergence between dwarf and normal lake whitefish species, 454 sequencing was used with the aim to generate a set of SNP markers, 89 SNPs showed pronounced allele frequency differences between sympatric normal and dwarf whitefish (Renaut et al., 2010).

Comparisons of Gene Expression Profiling Techniques

Hybridization-based approaches represented by microarrays are currently most popular for gene expression profiling and are readily affordable for many laboratories. Various commercial and academic microarray platforms have been developed that vary in genome coverage, availability, specificity, and sensitivity (e.g., Affymetrix, Agilent, and NimbleGen). Microarray approaches are high throughput and relatively inexpensive. However, these methods have several limitations, including relying on prior knowledge about genome sequence; high background levels owing to cross-hybridization (Royce et al., 2007); and a limited dynamic range of detection because of both background and saturation of signals. Moreover, comparing gene expression levels across different microarray experiments is often difficult and can require complicated normalization methods (Wang et al., 2009), although metagenomic analysis is possible.

In contrast to microarrays, direct sequencing of cDNAs was a digital method for gene expression measurement by counting mRNA molecules in the sample. Sequencing of cDNA or EST libraries was initially conducted using Sanger sequencing technology (Gerhard et al., 2004), but Sanger sequencing is relatively low throughput, expensive, and laborious. Therefore, EST analysis using Sanger sequencing for gene expression profiling is no longer a good choice.

More recently, RNA-Seq has become an alternative to microarrays (Wang et al., 2009). RNA-Seq provides many advantages over the traditional tag-based transcriptome analysis or microarray analysis including (1) Similarity between traditional SAGE analysis and MPSS, where RNA-Seq does not require any prior knowledge of genome sequence information; (2) Its extremely high throughput greatly improves the coverage of the transcriptome. Currently, Illumina HiSeq 2000 can generate over 200 million reads per lane, and the reads can be increased three times more using the newest chemistry, i.e., over 600 million reads per lane, allowing capture of the vast majority of transcriptome including many of the rarely expressed transcripts; (3) It overcomes many of the shortcomings of microarrays such as biases introduced during hybridization of microarrays; (4) Its cost is relatively low. One lane of RNA-Seq costs approximately $4000 while the construction of a comprehensive microarray often costs more; (5) The high throughput of reads allow technical assembly of sequences into contigs for additional studies such as gene structures. However, such a strength is also a weakness as assembly of RNA-Seq reads without reference genome or transcriptome poses challenges. De novo assembly, in particular, requires a greater level of bioinformatic expertise.

While microarrays have limitations for in-depth gene expression analyzes, they have the advantage being very useful for the high throughput analysis of multiple samples (Reinartz et al., 2002). Therefore, it may be helpful to consider the microarray and RNA-Seq as being complementary in nature which can be used as different tools for different types of experiments. For instance, to generate in-depth and quantitative gene expression data for species lacking genome information such as most of aquaculture species, RNA-Seq would be the best technology of choice. After the generation of expressed sequence data, it may be

necessary to examine whether sets of genes are differentially expressed in a large number of samples (e.g., individual gene expression variation) or under different conditions (e.g., different treatments), microarray analysis may be a better choice in terms of results and costs.

Functional Correlation of Gene Positions

In addition to expression correlation, gene functions can also be inferred from correlations of gene positions with the traits that are genetically also mapped to the same genomic location. In order to locate the positions of genes that are responsible for a certain trait, GWAS can be conducted. GWAS is a quantitative approach to analyze the association of whole genome DNA polymorphisms and a phenotypic trait, thereby localizing the genes underlining the trait.

Genome-Wide Association Studies (GWAS)

GWAS is a holistic whole-genome approach to robustly determine the association of DNA polymorphisms with correlated phenotypic traits. Most often, GWAS requires use of genome-wide polymorphic markers such as SNPs and at least hundreds of individuals with the phenotype information. We must stress the number of markers used because the more the markers, the better the markers over the entire genome; we also must stress the number of individuals because use of fewer than 200 individuals does not provide the confidence for the association. Many scientists feel that SNP association studies that are conducted on fewer than 200 animals that do not have either a confirmation population or functional data to support the association data are not reliable (e.g., James Reecy, Iowa State University, personal communication). The basis for GWAS analysis is that on a genome scale, most SNPs are distributed randomly in relation to the trait of interest, and only those SNPs tightly linked with the genes underlining the traits are in linkage disequilibrium (LD) in relation to the trait. GWAS usually involve tens or hundreds of thousands of SNPs that are tested on hundreds or thousands of individuals. These studies normally compare the DNA of two groups of participants: individuals with the trait, e.g., resistant fish, and individuals without the trait, e.g., susceptible fish.

GWAS has been extensively used for human disease research. For instance, in 2005, an association was found between age-related macular degeneration (ARMD) and a variation in the gene for complement factor H (CFH). Complement is a protein that regulates inflammation. Use of variation in the CFH gene, along with four other variants, can predict half the risk of ARMD between siblings, and this work was regarded as among the most successful examples of GWAS (Klein et al., 2005). Similarly, a GWAS involving genotyping of around 400K SNPs in a French case–control cohort allowed detection of an association between Type 2 diabetes and a variation in several SNPs in the genes TCF7L2, SLC30A8 and others (Sladek et al., 2007), which can explain a substantial portion of disease risk. In 2007, the Wellcome Trust Case Control Consortium carried out a GWAS of 14,000 cases of seven common diseases including coronary heart disease, Type 1 diabetes, Type 2 diabetes, rheumatoid arthritis, Crohn's disease, bipolar disorder, and hypertension. This study was successful in uncovering many new disease genes underlying these diseases (The Wellcome Trust Case Control Consortium, 2007).

In spite of being very powerful, GWAS has not been applied to aquaculture species. The primary reason was the lack of genome-wide polymorphic markers until recently. Now that a large number of SNPs are available for a number of aquaculture species, future application of GWAS in aquaculture species is clearly technically feasible. However, challenges related to low funding with aquaculture species are still

paramount as genotyping of a large number of polymorphic markers with a large number of individuals is expensive.

The problems associated with functional inference based on genomic positions come from the inaccuracy of GWAS analysis. On the one hand, the genomic location that are "in suspicion" to be involved in the trait can still involve large genomic segments, e.g., millions of base pairs that include many genes within the segment. On the other hand, GWAS may point to several or even many genomic locations for the trait of interest, complicating further functional analysis.

Analysis of Quantitative Trait Loci (QTL)

QTL analysis reveals statistically significant linkage between phenotypes and genotypes, thereby providing explanation for the genetic basis of variation in complex traits (Falconer and Mackay, 1996; Lynch and Walsh, 1998). In a sense, QTL analysis can be viewed as "incomplete" GWAS analysis with limited number of markers that does not cover the entire genome. As such, if one or few QTLs are found, there may be more QTLs in the genome to be discovered. More importantly, in the absence of closely linked markers in the genomic regions containing significant QTLs for the trait, the most significant genes responsible for the trait can be missed. However, because of historical reasons such as the lack of genome-wide markers, or the lack of funding, QTL analysis is still very important for aquaculture.

Many QTL studies have been conducted with aquaculture species. Most of these studies were conducted in salmonids. Table 1.1 lists some examples of QTL studies of aquaculture species.

For functional inference, QTL analysis provides limited power, as the resolution is low. However, in some cases, fine mapping of QTLs has allowed identification of causation genes or candidate genes for important aquaculture traits (see Table 1.1 for references).

Expression Quantitative Trait Loci Analysis

Studies have shown that mRNA levels for many genes are inheritable, thus amenable to genetic analysis (Brem et al., 2002; Cheung et al., 2003; Schadt et al., 2003). In the past few years, genetic and gene expression approaches have been brought together, in what has been coined "genetical genomics," to study the genetic basis of gene expression (Jansen and Nap, 2001), or expression quantitative trait loci (eQTL).

In the context of eQTL, gene expression, as measured by transcript abundance, is considered as a quantitative trait or phenotype, and in combination with genetic markers spaced across the genome, QTL are deciphered that account for variation in gene expression (Jansen and Nap, 2001). In other words, eQTL are genomic loci that regulate expression levels of mRNAs or proteins (http://en.wikipedia.org/wiki/Expression_quantitative_trait_loci-cite_note-0; Consoli et al., 2002).

Expression traits differ from most other classical complex traits in one important respect that the measured mRNA or protein trait almost always is the product of a single gene with a specific chromosomal location. eQTLs can be in either *cis* or *trans* with respect to the gene of interest. *cis*-Acting QTLs that map to the approximate location of their gene-of-origin are sequences flanking the gene (e.g., the promoter region) that regulate gene expression or transcript stability. On the other hand, *trans*-Acting QTLs are thought to involve transcription factors or other modulators, which map far from the location of their gene-of-origin, and can be on different chromosomes (Hansen et al., 2008).

eQTL analysis is the straightforward integration of traditional linkage analysis and global gene expression profiling. Both gene expression data and DNA marker data are collected in tissue samples from genetically related individuals. The expression level of each of the thousands of genes is treated as a separate quantitative trait, just like traits such as body weight

Table 1.1 Some examples of QTL studies of aquaculture species.

Species	Traits	References
Atlantic salmon	Body weight and condition factor	Reid et al., 2005
Atlantic salmon	Adaptive traits	Boulding et al., 2008
Atlantic salmon	Growth	Baranski et al., 2010
Atlantic salmon	Resistance against IPV	Houston et al., 2008; Moen et al., 2009; Gheyas et al., 2010; Houston et al., 2010
Atlantic salmon	Resistance against ISA	Moen et al., 2007
Atlantic salmon	Flesh colour	Baranski et al., 2010
Atlantic salmon	Life history	Vasemagi et al., 2010
Coho salmon	Hatch timing, weight, length and growth	McClelland and Naish, 2010
Rainbow trout	Upper thermal tolerance	Jackson et al., 1998; Danzmann et al., 1999; Perry et al., 2001; Perry et al., 2005
Rainbow trout	Life history	Leder et al., 2006
Rainbow trout	Spawning time	O'Malley et al., 2003; Colihueque et al., 2010
Rainbow trout	Osmoregulation capacities	Le Bras et al., 2011
Rainbow trout	Development rate	Robison et al., 2001; Easton et al., 2011
Rainbow trout	Whirling disease resistance	Baerwald et al., 2011
Rainbow trout	Growth	Wringe et al., 2010
Rainbow trout	Smoltification	Nichols et al., 2008
Arctic charr	Body weight, condition factor and age of sexual maturation	Moghadam et al., 2007; Kuttner et al., 2011
Arctic charr	Salinity tolerance	Norman et al., 2011
Asian seabass	Growth	Wang et al., 2006, 2011
European seabass	Body weight, morphometric traits and stress response	Massault et al., 2010
Gilthead Sea bream	Sex determination and body growth	Loukovitis et al., 2011
Tilapia	Sex determination	Shirak et al., 2006; Cnaani et al., 2007
Common carp	Muscle fiber-related QTL	Zhang et al., 2011
Eastern oyster	Disease resistance	Yu and Guo, 2006
Pacific Oyster	Growth	Guo et al., 2011
Pacific oyster	Resistance against summer mortality	Sauvage et al., 2010
Zhikong scallop	Size-related traits	Zhan et al., 2009
Pacific abalone	Growth-related traits	Liu et al., 2007
Blacklip abalone	Growth	Baranski et al., 2008

or disease resistance. The genomic loci that affect steady-state levels of each transcript are then determined by conventional QTL analysis. In this case, a significant QTL means that different genotypes at a polymorphic marker locus are associated with different expression levels. With the availability of an assembled genome sequence and genome-wide DNA microarray, this approach could be more powerful, simpler to implement, and used for any quantitative trait (Wayne and McIntyre, 2002). The advent of high-throughput array-based

methods to measure mRNA abundance in the early 2000s catalyzed an impressive number of expression QTL studies in plants, animals, and humans (Schadt et al., 2003; Morley et al., 2004; Bystrykh et al., 2005; Chesler et al., 2005; Hubner et al., 2005; Lan et al., 2006). Because RNA-Seq can provide the more accurate assessment of expression, a recent suggestion is to extend this technology to studies in eQTL analysis (Majewski and Pastinen, 2011). RNA-Seq could provide a platform independent and objective standard compared with the microarray approach. Recent eQTL studies using RNA-Seq technology have both confirmed and further clarified previous microarray results (Montgomery et al., 2010; Pickrell et al., 2010).

Mapping eQTLs is conducted using standard QTL mapping methods that examine the linkage or association between variation in expression and genetic polymorphisms. The only special consideration is that eQTL studies can involve a million or more expression microtraits. Genomic regions with a high proportion of eQTL could represent areas with genes that have common transcriptional regulators, which control important biological pathways (Cogburn et al., 2007). The ability to identify *trans*-Acting loci is particularly attractive because it is difficult to identify expression regulators even with a complete genome sequence. Standard gene mapping software packages can be used, although it is often faster to use custom code such as QTL Reaper or the web-based eQTL mapping system GeneNetwork (Wang et al., 2003; Wu et al., 2004). GeneNetwork hosts many large eQTL mapping data sets and provide access to fast algorithms to map single loci and epistatic interactions. As is true in all QTL mapping studies, the final step in defining DNA variants that cause variation in traits is usually difficult and requires additional rounds of experimentation. This is especially true for *trans* eQTL that do not benefit from the strong prior probability that relevant variants are in the immediate vicinity of the impacted gene. Statistical, graphical, and bioinformatic methods are used to evaluate positional candidate genes and entire systems of interactions (Lee et al., 2009).

Despite successes in other species, eQTL studies have not been conducted with aquaculture species due largely to the lack of technology in the past. However, it is important to point out that eQTL studies can be very expensive, and therefore, may not be suited well for aquaculture species. Even for the future, some caution should be considered before implementing eQTL analysis for aquaculture, similarly as had been pointed out for poultry (Cogburn et al., 2007). First, only one or a few tissues and time points are monitored in an individual, which provides a limited snapshot of the complete transcriptome. This must be kept in mind when results are interpreted (Cogburn et al., 2007). Second, how to properly analyze the data can be a significant challenge (Gibson and Weir, 2005). Current methods are limited in their ability to handle nonadditive, epistatic, or other complex gene effects. Third, experimental designs that do not have sufficient statistical power can compound the analysis. Only genes with large expression variation can be mapped because of the limitation of statistical power (Cogburn et al., 2007). It is widely recognized that gene expression measurements are subject to "noise," but without biological and technical replicates, one cannot determine the source of the problem. With regard to this issue, readers are referred to an excellent review by Rosa et al. (2006) for experimental design strategies for using microarrays in genetical genomics studies. Related to this limited statistical power is the ability to determine whether *cis*-Acting QTL are truly *cis*, where gene expression is regulated by the gene sequence itself, or the inability to separate out *trans*-Acting eQTL, which are closely linked (Cogburn et al., 2007). Finally, a large eQTL analysis project with a large number of genotyping and microarray assays represents an expensive venture that is not affordable for most laboratories working with aquaculture species. Although new and improved technologies with lower costs per data

point are available, the cost of genotyping and genome-wide transcriptional profiling needs to be reduced for implementation in aquaculture.

Functional Inference by Comparative Genome Analysis

A variation of position-based functional inference is functional inference through comparative genome analysis. With this approach, gene orthologies and conserved genome syntenic regions are first identified between the species of interest with well-studied model species. If the functions of a specific gene were well characterized in the model species, by inference, the orthologous gene would have the same or similar functions in the species under study. Given the large numbers of genes and the difficulties involved in determining the functions of genes, this approach could be the most popular approach for aquaculture species for the vast majority of genes. Genome-wide comparative analysis enables the transfer of genome annotation from better-annotated species to a newly-sequenced, related species. However, caution need to be exercised because: (1) Gene functions may have evolved through evolution such that the orthologous gene now may have different functions; (2) for important aquaculture traits, there would be no known "functional equivalent" in the model species. For instance, the enteric septicemia disease of catfish exists only in catfish, and it would become obscure as to what genes we should look after in zebrafish for potential candidate genes.

Theoretical Basis for Functional Inference Based on Orthologies

The evolution of life on earth, which appears to have begun billions of years ago, is recorded in the genome of modern organisms. Since the organisms are more likely to be negatively affected by mutations in functional regions, the genes and other functional elements undergo mutation at a slower rate than the rest of the genome. Therefore, the genomes of current organisms inherited from their ancestral counterparts preserve conservation because of the evolutionary pressure. Based on this principle, comparative genomics approaches have been applied to not only study the evolution of genome, but also to expand our knowledge of biological processes across multiple species by projecting high-quality annotations from one genome to another. This is a promising direction in current functional genomics research as all the genomes of many different species are being sequenced. For instance, researchers have learned a great deal about the function of human genes by examining their counterparts in simpler model organisms such as the mouse (Lee et al., 2004; Crozat et al., 2010). Because the research resources invested in model species are far greater than those invested in aquaculture species, transferring the genome information from well-characterized species can significantly advance our knowledge in aquaculture species. Comparative genomics approaches have been proven to be very effective in identifying homologous genes and functional elements in complex genomes (Cogburn et al., 2007). With the aid of actively developed bioinformatics tools, genome sequence similarity, chromosomal location and organization, and conserved sequences are integrated to effectively detect and annotate novel genes in newly-studied organisms, such as aquaculture species.

Conserved Synteny and Orthology

An important concept of comparative genomics is conserved syntenies, i.e., conserved gene sequences and their chromosomal organization. During the course of evolution, chromosome rearrangement, gene duplication, gene divergence, and gene loss have occurred. However, in vertebrates, gene arrangement in chromosomes is usually conserved, and can be classified into conserved syntenies. Strictly speaking, conserved syntenies are conserved segments that are uninterrupted by other chromosomal

segments, and conserved order of genes in the same linear orientation on the chromosome (Andersson et al., 1996). However, in many cases, as the genomic region of conserved segments extends, the exact gene order and orientation of genes may vary among related species (Eichler and Sankoff, 2003; Hurst et al., 2004).

Orthology refers to a gene in two or more species that has evolved from a common ancestor. As evolutionary studies are often from inference rather than tested by experiments, the evolutionary origin of a gene is mostly inferred from conserved syntenies.

For functional inference, if orthologies can be established, and assuming the orthologous genes have similar functions in related species, then functions of a gene in aquaculture species can be inferred from its closely-related model species. In this regard, the fish model zebrafish is highly useful because many genes can be tested on their functions.

Another application of comparative genomics is to identify regulatory sequences that control gene expression (Cogburn et al., 2007). Regulatory sequences are *cis*-Acting modules (i.e., promoters and enhancers) that govern the spatiotemporal expression of genes. Identification of regulatory sequences in the genome is far more difficult than prediction of genes. In the absence of systematic biochemical analysis, the prediction of functional elements in the genome of a new organism depends heavily on computational and comparative analysis (Jones and Pevzner, 2006). Genome sequences harboring these functional elements seem to be conserved among related species. The alignment of genome sequences of several related species allows the identification of evolutionary conserved sequences containing transcription regulatory elements. Comparative analysis of the human with mouse, rat, and dog genomes sequences have identified many common regulatory motifs in human promoters and 3′-untranslated regions (Xie et al., 2005). Various computational tools have been developed and assessed for the efficiency in predicting transcription factor-binding site (TFBS) motifs in genome sequence (Tompa et al., 2005). However, because of the nature of the regulatory sequences being short, species-specific and variable, not all TFBS can be identified by comparative or computational approaches unless accompanied by robust statistical confirmation and experimental validation (Elnitski et al., 2006; GuhaThakurta, 2006). The most reliable approach to detect regulatory regions with transcription factor binding is ChIP-chip analysis (Horak and Snyder, 2002; Valouev et al., 2008), and more recent ChIP-Seq which allowed genome-wide detection of TFBS with unprecedented sensitivity and specificity (Hu et al., 2010; Schmidt et al., 2010).

Gene Pathway Analysis

In order to effectively mine EST, microarray, or RNA-Seq data for meaningful biological information, gene set enrichment analysis (GSEA) has been recently adopted to provide clues as to what gene pathway may be turned on or turned off under a specific treatment (e.g., He et al., 2011; Kim et al., 2011b; Zhang et al., 2011). A number of pathway analysis software packages are available such as Pathway Studio (http://www.ariadnegenomics.com/), and MetaCore™ (http://www.genego.com/metacore.php). In such software packages, the algorithms calculate the statistical significance of the expression changes across every group or pathway in the database, thus, allowing identification of groups or pathways most strongly affected by the observed expression changes (http://www.ariadnegenomics.com/technology-research/pathway-analysis/). In simple terms, such software finds the genes mostly up- or down-regulated, and then places them into the gene pathways these genes are involved in, and then pins down the gene pathways that are operating under the specific condition of the "treatment."

Although gene pathway analysis has been fruitful for studies involving mammals, it has

not yet been extensively used in aquaculture species. Recently, a few studies were conducted with fish (Olsvik et al., 2008; Wang et al., 2010; Sanchez et al., 2011; Thomas et al., 2011). These studies demonstrated the usefulness of GSEA for functional genomics analysis in aquaculture species.

Profiling of DNA–Protein Interactions and Epigenetic Modifications

The control of gene expression mostly occurs at the level of transcription, so information on genome-wide chromatin profiles and DNA–protein interactions is essential to decipher the inherent logic of transcriptional regulation (Schones and Zhao, 2008). The study of epigenetics focuses on heritable changes in gene expression that does not involve the underlying DNA sequence but the epigenetic modifications such as DNA methylation and histone modification. Recent research has implicated the importance of epigenetic modifications in development, cell differentiation, and oncogenesis, setting the grounds for the Human Epigenome Project (HEP) initiative, which aims to catalog DNA methylation patterns on a genome-wide scale (Esteller, 2006). Genome-wide mapping of epigenetic modifications and protein–DNA interactions is essential for a full understanding of transcriptional regulation, and there is a growing recognition that systematic profiling of the epigenomes in multiple cell types and stages may be needed for understanding developmental processes and disease states (Bernstein et al., 2007).

The main approach for investigating epigenetic mechanisms is chromatin immunoprecipitation (ChIP), which is a technique for assaying protein–DNA binding in vivo (Solomon et al., 1988). In ChIP, antibodies are used to select specific proteins or nucleosomes, which enriches for DNA fragments that are bound to these proteins or nucleosomes (see illustrations in Figure 1.3). The introduction of microarrays allowed the fragments obtained from ChIP to be identified by hybridization to a microarray (ChIP–chip), therefore enabling a genome-scale view of DNA–protein interactions (Ren et al., 2000). Briefly, chromatin fragments are isolated using antibodies that are specific to a particular histone modification and the isolated fragments are amplified to generate micrograms of fluorescently labeled DNA, which is subsequently hybridized to DNA microarrays.

The rapid technological developments in next-generation sequencing enabled the combination of ChIP with massively parallel sequencing (ChIP–Seq), which allows researchers to survey more of the genome in less time and promises to unveil new aspects of biology (Johnson et al., 2007; Robertson et al., 2007). In ChIP–Seq, the DNA fragments of interest are sequenced directly instead of being hybridized on an array. Briefly, chromatin fragments are isolated using antibodies that are specific to a particular histone modification, and the isolated ChIP DNA is ligated to a pair of adaptors and subjected to high throughput sequencing. The number of sequenced reads mapped to a genomic locus is directly proportional to its modification level. In contrast to ChIP-chip, ChIP-Seq requires less PCR amplification and does not rely on the efficiency of probe hybridization, it is probably more quantitative and the modification levels at different genomic regions can be directly compared (Schones and Zhao, 2008).

Application of these techniques has led to great advances in our understanding of how epigenetic phenomena are regulated and how they affect gene expression. Epigenetic studies are currently solely limited to human and some other model species such as mouse, fruit fly, and yeast. However, the advances of high throughput microarray and next-generation sequencing can make these techniques feasible to decipher epigenetic mechanism in aquaculture species. In the following text, we give a summary of the applications of these techniques in model species. Interested readers are referred to the excellent reviews published in the last

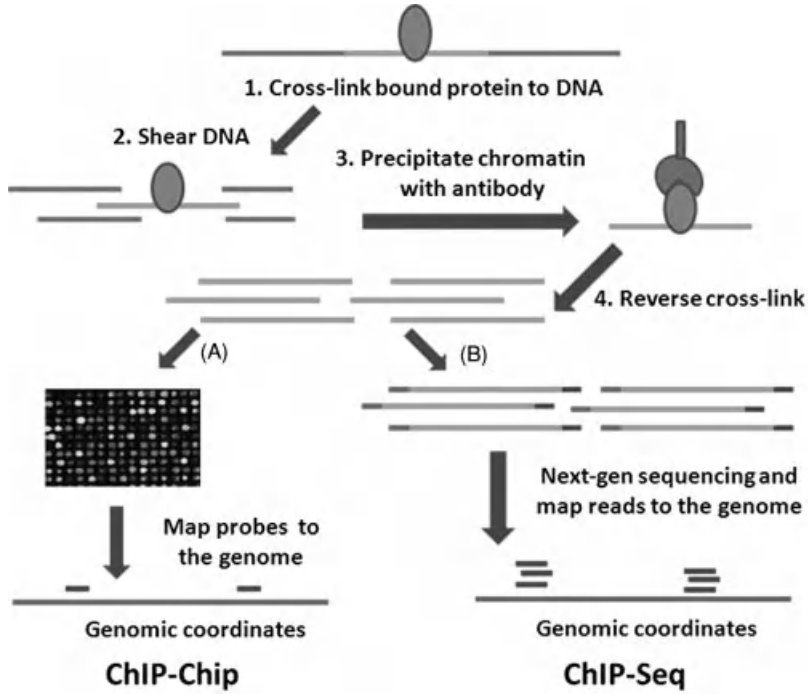

Figure 1.3 A schematic presentation of Chromatin immunoprecipitation (ChIP) combined with microarray (ChIP-Chip) and high-throughput sequencing techniques (ChIP–Seq). The first step of ChIP is to cross-link bound proteins to DNA, then isolate the chromatin and shear DNA, precipitate chromatin with protein-specific antibody, and lastly, reverse cross-link and digest protein to release the bound DNA. In ChIP-Chip (left lower panel), the ChIP DNA is then amplified to obtain sufficient DNA followed by hybridization to a DNA microarray. The microarray probes can then be mapped to the genome to yield genomic coordinates; in ChIP–Seq (right lower panel), the ChIP DNA ends are repaired and ligated to a pair of adaptors, followed by high throughput sequencing. The resulting sequence reads are mapped to a reference genome to obtain genomic coordinates that correspond to the immunoprecipitated fragments. See color insert.

several years (Ren et al., 2000; Holliday, 2006; Goldberg et al., 2007; Henikoff, 2008; Schones and Zhao, 2008; Suzuki and Bird, 2008; Park, 2009; Laird, 2010).

DNA Methylation

DNA methylation is defined as the addition of a methyl group to the cytosine in a CpG dinucleotide. This covalent modification is known to have an important role in genome function, such as gene regulation, chromosomal stability, and parental imprinting (Bird, 2002). CpG islands, GC-rich regions within mammalian genomes, are resistant to methylation and are associated with most human genes (Ng and Bird, 1999). Gene inactivation can be observed by de novo methylation of promoters that contain CpG islands (Bird, 2002), and DNA methylation of TBFS can influence their binding (Tate and Bird, 1993; Bell et al., 1999).

Studies of DNA methylation on a genome scale primarily rely on "local" techniques combined with global approaches such as DNA microarrays or high-throughput sequencing. The most important local techniques include genomic mapping of cleavage sites by restriction enzymes that differentiate between methylated and unmethylated CpG DNA sequences. Sequencing of DNA after treatment with sodium

bisulphite, which converts all unmethylated cytosines to uracils, allows differentiation of methylated and unmethylated sites. Affinity purification can be used to isolate protein-bound DNA with methylcytosine DNA-binding domain (MBD) proteins, and immunoprecipitation of DNA with an antibody that recognizes 5-methyl cytosine (known as MeDIP, mDIP or mCIP) (Schones and Zhao, 2008). In the past few years, immunoprecipitation of methylated DNA has been combined with microarrays to study DNA methylation at a genome scale in human cells (Weber et al., 2005; Keshet et al., 2006). Recently, MeDIP has been combined with tiling microarrays to provide a DNA methylation map of the *Arabidopsis thaliana* genome (Zhang et al., 2006; Zilberman et al., 2007), and with high-density promoter arrays to map the human promoter methylome (Shen and Waterland, 2007; Weber et al., 2007).

Histone Modifications

Histone proteins play important roles in gene regulation process. In general, genes those are active have less bound histones, while inactive genes are highly associated with histones during interphase. Histone proteins are subject to a number of covalent post-translational modifications, primarily at their N-terminal tails, including methylation, acetylation, phosphorylation, ubiquitylation, and ADP-ribosylation. Histones undergo post-translational modifications that alter their interaction with DNA and nuclear proteins, which affect their function of gene regulation (Schones and Zhao, 2008).

The most prevalent technique used to map histone modifications at a genomic scale has been the combination of ChIP with DNA microarrays (ChIP-chip). The first ChIP-chip studies of histone modifications in *Saccharomyces cerevisiae* (Bernstein et al., 2002; Robyr et al., 2002) and *Drosophila melanogaster* (Schubeler et al., 2004) suggested that histone modifications are associated with distinct genomic regions and with distinct transcription states. Subsequent studies with higher resolution tiling arrays in yeast (Liu et al., 2005; Pokholok et al., 2005) reinforced the concept of redundancy in histone-modification maps. ChIP-chip has also been used to profile histone modifications in mammalian genomes (Bernstein et al., 2005; Heintzman et al., 2007).

More recently, ChIP-Seq is used to profile histone modifications. The first applications of ChIP-Seq were done in $CD4^+$ T cells (Barski et al., 2007) and mouse embryonic stem (ES) cells (Mikkelsen et al., 2007).

Numerous genome-wide profiles of histone modifications have been conducted in various model organisms. In studies using yeast, the histone H3 lysine 4 methylation (H3K4me) and histone acetylation were found positively correlated with transcription levels, and are highly enriched in promoter regions and extend into the transcribed regions significantly (Liu et al., 2005; Pokholok et al., 2005). A general concept is now emerging in which distinct genomic regions (e.g., enhancers, promoters and genes) have distinct histone-modification patterns (Heintzman et al., 2007). Genome-wide distribution patterns of histone acetylation have allowed successful identification of functional enhancer elements (Roh et al., 2007). Novel transcription units and functional transcription start sites have also been determined based on modification patterns (Barski et al., 2007). It has become apparent that histone modification patterns can be a useful means to facilitate a more precise annotation of the human and other genomes.

DNA–Proteins Interactions

Gene expression is often regulated by proteins that activate or repress transcription by binding to short, specific DNA sequences. Such *cis*-Acting sites are usually located close to the promoter (TFBS) for the regulated gene. In a ChIP-Seq experiment for DNA-binding proteins, DNA fragments associated with a specific protein are enriched and sequenced.

The earliest reports using ChIP-Seq focused on identification of DNA-binding sites for the transcription factors NRSF (neuron-restrictive silencer factor) (Johnson et al., 2007) and STAT1 (Signal transducer and activator of transcription 1) (Robertson et al., 2007). Genome-wide mapping of many transcription factors has now been reported (Jothi et al., 2008; Valouev et al., 2008; Wederell et al., 2008). Many of these studies show that TBFS are found upstream of the transcription start site, downstream of the termination signal, within introns and exons of genes and in genomic regions far away from any annotated genes. Such diversity in DNA-binding site locations highlights the power of genome-wide analysis and our need to better understand mechanisms of gene regulation. As thousands of DNA-binding sites are normally found for each transcription factor, determining the functions of protein–DNA interactions can be difficult. Gene expression profiling can be used to determine whether the associated gene is expressed in the cells or tissues. In addition, patterns of specific histone modification around TFBS can be used to evaluate whether the site is acting to activate or repress transcription (Heintzman et al., 2007; Koch et al., 2007). By combining transcription factor binding analysis with gene expression profiling and histone modification, a detailed picture of binding site activity can emerge (Cullum et al., 2011).

Direct Test of Gene Functions

Traditional Approaches

Gene functions can be directly tested with forward genetics or reverse genetics approaches. In forward genetics, the phenotype is already known, usually from a mutant with visible or measurable phenotype. The genes responsible for the mutation can be mapped and characterized. The basic concept of the forward genetics approaches is that if a mutant phenotype is mapped to a locus where a gene mutation is found, then the gene harboring the mutation is responsible for the mutant phenotype. In this way, functions of many genes have been determined. In zebrafish, mutant libraries have been created to study the genes responsible for the mutation (e.g., Ellett and Lieschke, 2010; Lessman, 2011; Peal et al., 2011). However, its limitation is the inability for generation of mutants with a visible phenotype, particularly for aquaculture traits. Functional genomics using forward genetics approaches are well reviewed by Hackett and Clark (2007), and interested readers are referred to his review.

Reverse genetics approaches have also been used for the study of gene functions. Transgenic technology and knockout/knockdown technologies are among the most used approaches. With transgenic technology, enhanced growth rate was clearly demonstrated with the transfer of growth hormone genes (Du et al., 1992; Gross et al., 1992; Devlin et al., 1994; Rahman et al., 1998; Rahman et al., 2001). The basic concept of functional analysis through transgenesis is that if a gene has a specific function, then the introduction of the gene or an increase in its copy numbers in the genome should generate a noticeable phenotypic change. While transgenic technology is very effective for the analysis of gene functions, genome-wide test of functions of many genes may be difficult because the inefficiency of transgenic technology, screening for transgenic animals, and the associated time, efforts, and costs.

There are still no effective knockout systems in aquaculture species, but knockdown using RNAi and morpholinos have been conducted in many studies (e.g., Huang et al., 2008; Anastasaki et al., 2011). For instance, RNAi was employed to knockdown three subunits of the gonadotropin alpha (GtHalpha), follicle-stimulating hormone beta (FSHbeta), and luteinizing hormone beta (LHbeta) genes. Expression of GtH mRNA was obviously and more efficiently depressed by GtHalpha RNAi expression. A GtHalpha morpholino analysis showed that the GtHalpha morpholino led to

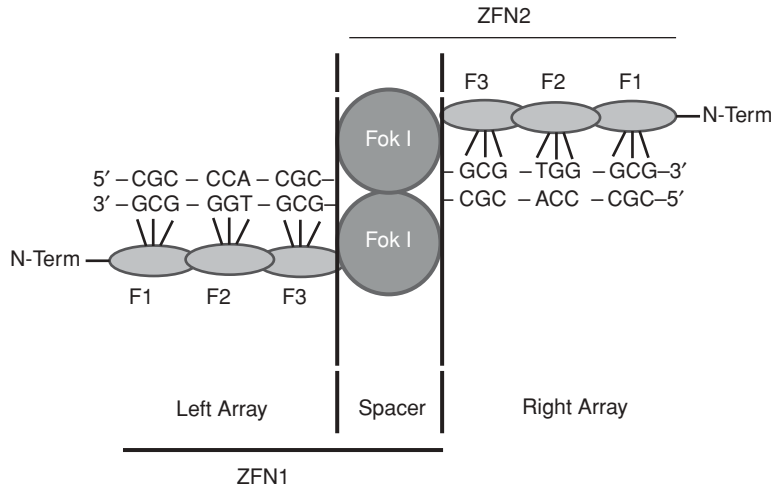

Figure 1.4 Presentation of engineered zinc finger nuclease (ZFN). The sequence specificity is determined by individual ZFN1 and ZFN2 fingers. The Fok I endonuclease domain introduces double-stranded breaks after sequence-specific DNA binding. (This figure was adopted from Dr. Stephen C. Ekker (2008) with his permission.) See color insert.

suppression of embryonic development and the production of embryonic mutants as a result of an injection of GtHalpha–shRNA (Huang et al., 2008). However, these approaches are by no means ideal for functional genomics studies in aquaculture species. The main problems of knockdown technologies are their inability to provide sufficient knockdown. The level of suppression of gene expression by these technologies may vary depending on the systems.

Zinc Finger Nuclease (ZFN) Technology

A recently developed technology, the zinc finger nuclease (ZFN) technology, enables targeted editing of the genome by introducing double-strand breaks in DNA at specific locations by taking advantage of ZFNs. ZFN technology shows a great promise for functional genomics research, including aquaculture species.

ZFN technology is based on engineered ZFNs. Engineered ZFNs are artificial restriction enzymes composed of zinc finger (ZF) DNA-binding domain and a DNA-cleavage domain (Kim et al., 1996). As depicted in Figure 1.4, the DNA-binding domain of ZFN determines the specificity, while the endonuclease Fok I cleave the DNA.

The ZF domains can be engineered to bind target DNA sequences of interest (Segal and Barbas, 2001; Beerli and Barbas, 2002), enabling the creation of double-strand DNA breaks (DSBs) at targeted genome locations. Repair of ZFN-induced DSBs can be a nonhomologous end-joining (NHEJ) event that is error-prone and leads to efficient introduction of insertion or deletion (indels) mutations at the site of DSB (Figure 1.5).

Alternatively, repair of a DSB by homologous recombination (HR) machinery with supplied DNA fragment as a template can promote efficient introduction of alterations or insertions at or near the break site (Sander et al., 2011)

With the popularity of ZFN technology, a ZFN Consortium has been established: The Zinc Finger (ZF) Consortium (http://www.zincfingers.org/scientific-background.htm). This Consortium is committed to developing

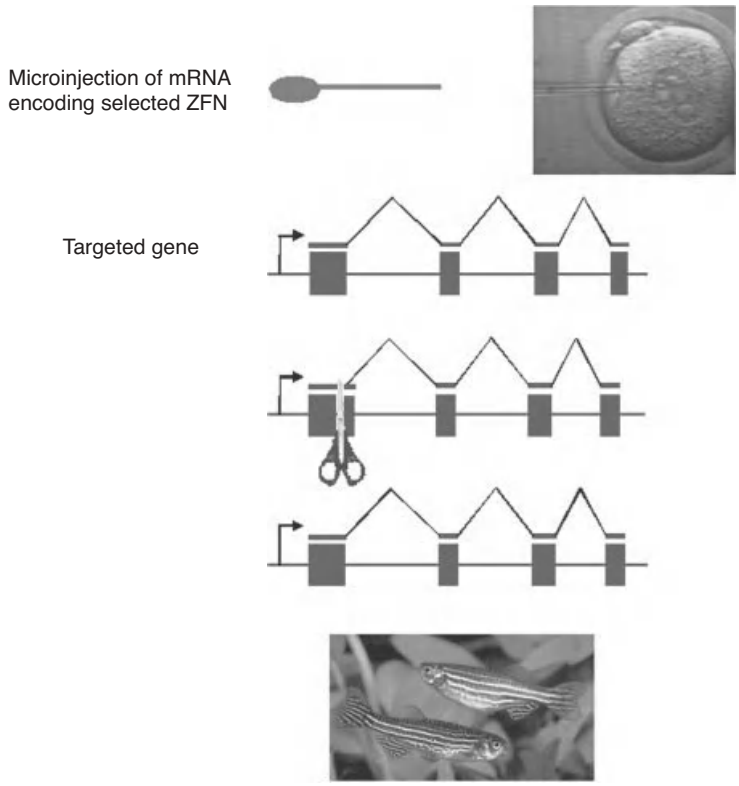

Figure 1.5 Schematic presentation of the processes of making a targeted gene knockout in zebrafish using ZFNs. mRNA encoding the selected ZFN is injected into one-cell zebrafish embryos. This custom ZFN binds and makes a double stranded break at the specified locus. Cell repairs the double stranded DNA break imprecisely to introduce mutations to the selected gene. The specific molecular lesion is clonally selected and determined after genotyping the offspring from these mosaic founders. (This figure is adopted from Dr. Stephen C. Ekker (2008) with his permission.) See color insert.

resources, software, and other tools for engineering ZFs and for performing genome engineering that are robust, user-friendly, and publicly available to the academic scientific community.

Approaches for engineering zinc-finger arrays were developed to allow widespread adoption and large-scale use of ZFN technology. As summarized by Maeder et al. (2008), zinc-finger engineering methods can be grouped into two general categories: The first is "Modular assembly," which involves joining together single fingers with pre-characterized specificities (Liu et al., 2001; Beerli and Barbas, 2002; Bae et al., 2003; Segal et al., 2003; Mandell and Barbas, 2006; Kim et al., 2011a). Modular assembly is easy to perform, but the success rate is relatively low (6%) (Ramirez et al., 2008), and can yield ZFNs with low activities and/or high toxicities (Cornu et al., 2008; Pruett-Miller et al., 2008). The second approach involves combinatorial selection-based methods that yield multi-finger domains possessing high DNA-binding affinities and specificities (Greisman and Pabo, 1997; Isalan et al., 1997, 2001; Hurt et al., 2003) and high activities and low toxicities when expressed as ZFNs in human cells (Cornu et al., 2008; Pruett-Miller et al., 2008). The weakness

of selection-based methods, as summarized by Maeder et al. (2008) is that it requires construction and interrogation of large numbers of randomized libraries (typically >108 in size). Recently, Maeder et al. (2008) developed a method known as OPEN (Oligomerized Pool ENgineering). The efficiencies of OPEN are high, ranging from 1%–50%. Apparently, the publicly available OPEN platform will enable routine practice and further development of ZFN technology.

Several software tools have been developed including ZiFiT, ZiFDB, and ZFNGenome. The ZiFiT software package identifies potential target sites in DNA sequences for which ZF proteins may be engineered (Sander et al., 2010a; 2010b; Sander et al., 2007). ZiFDB is a web-accessible database of ZFs and engineered ZF arrays, which organizes information on both individual ZF modules and engineered ZF arrays (Fu et al., 2009). ZFNGenome is a GBrowse-based tool for identifying and visualizing potential target sites of OPEN-generated ZFNs (Reyon et al., 2011).

ZFNGenome currently includes a total of more than 11 million potential ZFN target sites mapped within the fully sequenced genomes of seven model species.

ZFN technology can be used for gene knockouts. For instance, ZFN-induced knockouts have been created in the kdr gene in zebrafish (Meng et al., 2008). In another study, NHEJ-based repair of ZFN-induced DSBs were exploited to disrupt gol and ntl genes in zebrafish to generate mutants (Doyon et al., 2008). These results demonstrated the potential of this technology for reverse genetics applications. To date, no published data are available for use of ZFN technology in aquaculture species. However, heritable knockout lines of aquaculture fish species have been produced (Dr. Qingshun Zhao, Nanjing University, China, Personal communications).

Because homologous recombination relies on homologous DNA to repair the DSB, gene "editing" can be achieved by providing an exogenous "donor" template (Reyon et al., 2011). This results in replication of the "donor" DNA sequence at the specified locus. Many studies have taken advantage of this process to introduce small mutations or large insertions in plants and animals such as maize (Shukla et al., 2009), fruit fly (Beumer et al., 2006) and human (Zou et al., 2009).

Although most of previous ZFN works focus on model species, we can envision that ZFN technology will be applied to aquaculture species in the near future. The results derived from the applications of ZFN technology in zebrafish will absolutely offer prior knowledge for similar work in aquaculture fish species. At the same time, more and more genome sequencing projects of aquaculture species will provide the fundamental genomic resources for the identification of potential target site and the design of engineered ZF arrays.

Functional Genomics Approaches Suitable for Aquaculture

Currently, the whole genome projects have been "completed" in six fish species including Zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), *Tetraodon* (*Tetraodon nigroviridis*) Stickleback (*Gasterosteus aculeatus*), and Atlantic cod (*Gadus morhua*). With the advances in next-generation sequencing technology, more than 30 whole genome sequencing projects are underway for aquaculture species such as Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*), nile tilapia (*Oreochromis niloticus*), Half-smooth tongue sole (*Cynoglossus semilaevis*), channel catfish (*Ictalurus punctatus*), and many more. For shellfish and crustaceans, a number of species are also being sequenced such as Pacific oyster (*C. gigas*), zhikong scallop (*Chlamys farreri*), and Pacific white shrimp (*Litopenaeus vannamei*). Although none of these genomes has been completely finished to a stage that sequences are assembled together into chromosome, the huge-scale sequence resources

generated during this process are valuable for genome-scale functional genomics studies. For species lack of genome projects, a wealth of sequence data exist which are largely from cDNA sequencing projects (see Chapter 2 for EST and RNA-Seq resources generated in aquaculture species). Although all the technologies will continue to provide functional genomics information, in the following text, we attempt to make some assessment of the existing technology.

With the genome or transcriptome resources, microarrays can be readily developed for functional genomics research in aquaculture species. While situation may vary, we anticipate that microarrays will still have important roles to play for functional genomics analysis, especially for species with relatively larger research communities.

In spite of its historical contributions, EST analysis will become a limited approach because of its high cost. In contrast, the next-generation sequencing based gene expression analysis is increasingly feasible with the sequencing cost becoming lower and lower. We see that RNA-Seq can be a very rapid and technologically ready approach for aquaculture functional genomics, but large-scale application with many individuals may still be cost-prohibitive presently.

With the increasingly more availability of genome-scale SNP markers, the GWAS perhaps will become the most important approach among all for the determination of genes involved in performance and production traits important for aquaculture. Most aquaculture species have high fecundities with the ability to produce a large number of progenies in a single spawn, allowing genetic associations to be analyzed with minimal "noise" in the system. On the other hand, because of the aquatic living environment, phenotypes of aquaculture species are more difficult to measure, making GWAS more challenging as well. Traditional QTL analysis will continue to have an important role in providing basic information of genomic locations underlying various traits.

Although many eQTL studies have been conducted in plants and human as discussed in the previous text, few studies were conducted in aquaculture species. Recently, an eQTL analysis conducted on the dwarf and normal whitefish species pairs identified 253 genes differentially expressed in white muscle between these two coregonid ecotypes, 33 of which were associated with 53 eQTL (Derome et al., 2008). However, as discussed in the previous text, application of eQTL in aquaculture species will prove to be difficult, not because of technical reasons, but because of limitations of funding.

As the whole genome sequences are being assembled with aquaculture species, comparative genomics will perhaps be a very powerful approach for functional inference, simply because it is not possible to determine functions of genes in each species, related functions can be inferred one another based on evolutionary conservation of genes and their functions. In this regard, work in model species will continue to be useful for aquaculture species.

Epigenetics is a rapidly developing field of functional genomics because of its recognized role in determination of the phenotype. At present, the worm, fruit fly, and mouse are the most frequently used models in epigenetics. Epigenetic study in aquaculture is still in its infancy. Marbled crayfish (*Marmorkrebs*) was recently proposed as a model organism for research in epigenetics (Vogt, 2008). DNA methylation studied for marbled crayfish revealed that a global DNA methylation in the hepatopancreas and abdominal musculature varied from 1.52–1.94% and 1.77–2.09%, which corresponds to roughly half of the values in humans (Vogt, 2008). In spite of its importance, epigenetic studies in aquaculture species are generally limited by funding at present.

Among many of the direct experimental approaches for the determination of gene functions, the ZFN technology shows the greatest promise for use in aquaculture species. Clearly, investment should be made to develop ZFN technology and its application with aquaculture species.

Acknowledgments

Research in our laboratory is supported by grants from the United States Department of Agriculture (USDA) National Research Initiative (NRI) Animal Genome and Genetic Mechanisms Program, the USDA NRI Basic Genome Reagents and Tools Program, the Mississippi–Alabama Sea Grant Consortium, the Alabama Department of Conservation, the United States Agency for International Department (USAID), and the Binational Agriculture Research and Development Fund (BARD).

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Chapter 2
Genomic Resources for Functional Genomics in Aquaculture Species

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Abstract: Functional genomics studies require the availability of genome resources. In the last decade, huge sets of genome resources have been developed from aquaculture species. In this chapter, we provide a brief summary of what is available with various aquaculture species. Our guiding principle, however, is to provide some basic information such that any reader can follow up to find the required information rather than to provide a complete list of resources for all species. In this chapter, we provide an overview of existing genome resources for functional genomics research including DNA markers, expressed sequence tags (ESTs), microarrays, next-generation sequence read archive (SRA) databases, single nucleotide polymorphism (SNP) genotyping platforms, databases for aquaculture genome projects, and whole genome sequence assemblies.

Introduction

The goal of functional genomics research is to reveal gene functions on the genomic scale. With the large numbers of genes an organism has, genome-scale functional analysis can be quite complex. This is further complicated by involvement of many genes in a single function in many cases, multiple functions of a single gene (pleiotropy) in many cases, and interactions of gene products. As a result, functional genomics studies require a set of genome resources, without which effective functional genomics research is difficult. For instance, mapping of a trait or a phenotype would require polymorphic DNA markers such as microsatellites (SSRs) or single nucleotide polymorphisms (SNPs); expression profiling would require genome annotation information; microarray design would require sequence information of genes, etc. The objective of this chapter is to provide a general review of genomic resources needed, and currently present for aquaculture species, for functional genomics studies.

Polymorphic DNA Markers

The key factor behind the significant differences at the level of individuals, species, and higher order of taxonomic groups is genetic variation (polymorphism). These variations provide a species the ability of adapting to the environment change (Liu and Cordes, 2004). DNA markers are among the most powerful tools for revealing genetic variations in organisms.

Historically, many different types of markers have been used for aquaculture studies

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

Table 2.1 A summary of characteristics of various molecular markers used in aquaculture species.

Marker	Abbreviation	Prior molecular information requirement	Type	Polymorphism or power	Expression
Allozyme	–	Yes	Type I	Low	Codominant
Amplified fragment length polymorphism	AFLP	No	Type II	High	Dominant
Expressed sequence tags	EST	Yes	Type I	Low	Codominant
Insertions or deletions	Indels	Yes	Type I or Type II	Low	Codominant
Microsatellites	SSR	Yes	Mostly Type II	High	Codominant
Mitochondrial DNA	mtDNA	Yes	–	–	Maternal inheritance
Random amplified polymorphic DNA	RAPD	No	Type II	Moderate	Dominant
Restriction fragment length polymorphism	RFLP	Yes	Type I or Type II	Low	Codominant
Single nucleotide polymorphisms	SNPs	Yes	Type I or Type II	High	Codominant

including allozymes, mitochondrial DNA (mtDNA) markers, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), SSR, SNPs, and expressed sequence tag (EST) markers, etc. Some of the basic properties of these marker types are summarized in Table 2.1.

Currently, the most important markers for aquaculture research are SSRs and SNPs, and clearly the importance of SNPs is on the rise. In contrast, several marker types are no longer relevant for genome-scale studies. This trend is reflected in the literature that is generated using these markers. As shown in Figure 2.1, a search of ScienceDirect (http://www.sciencedirect.com/) apparently indicated that all the marker types, with the exception of SNPs, have reached their peak of applications. The use of several types of markers such as allozyme and RAPD markers is clearly on the decline (Figure 2.1).

Great efforts have been devoted to the development of DNA markers. Here, we are not attempting to provide a thorough review of such efforts, but provide a few examples. For instance, allozyme markers have been developed in common carp (K. Sumantadinata and Taniguchi, 1990; Desvignes et al., 2001), Atlantic salmon (Artamonova, 2007), Atlantic cod (Pogson et al., 1995), rainbow trout (Ferguson et al., 1993), and Mrigal carp (Singh et al., 2004), etc.; mtDNA markers have been used with Atlantic eels (Avise et al., 1986), red drum (Gold et al., 1993), Atlantic snappers (Chow et al., 1993), common carp (Chang and Huang, 1994), red grouper (Zhu and Yue, 2008), etc.; RAPD markers have been used in catfish (Liu et al., 1998), Atlantic salmon (Elo et al., 1997), Asian arowana (Yue et al., 2002), etc.; microsatellite markers have been widely used in Atlantic salmon (e.g., Slettan et al., 1997), catfish (e.g., Waldbieser and Bosworth, 1997; Liu et al., 1999a, 1999b), tilapia (e.g., Lee and Kocher, 1998; Palti et al., 2001; Carleton et al., 2002), rainbow trout (e.g., Rexroad et al., 2001, 2002), common carp (e.g., Tanck et al., 2001; Li et al., 2007a); SNPs have been

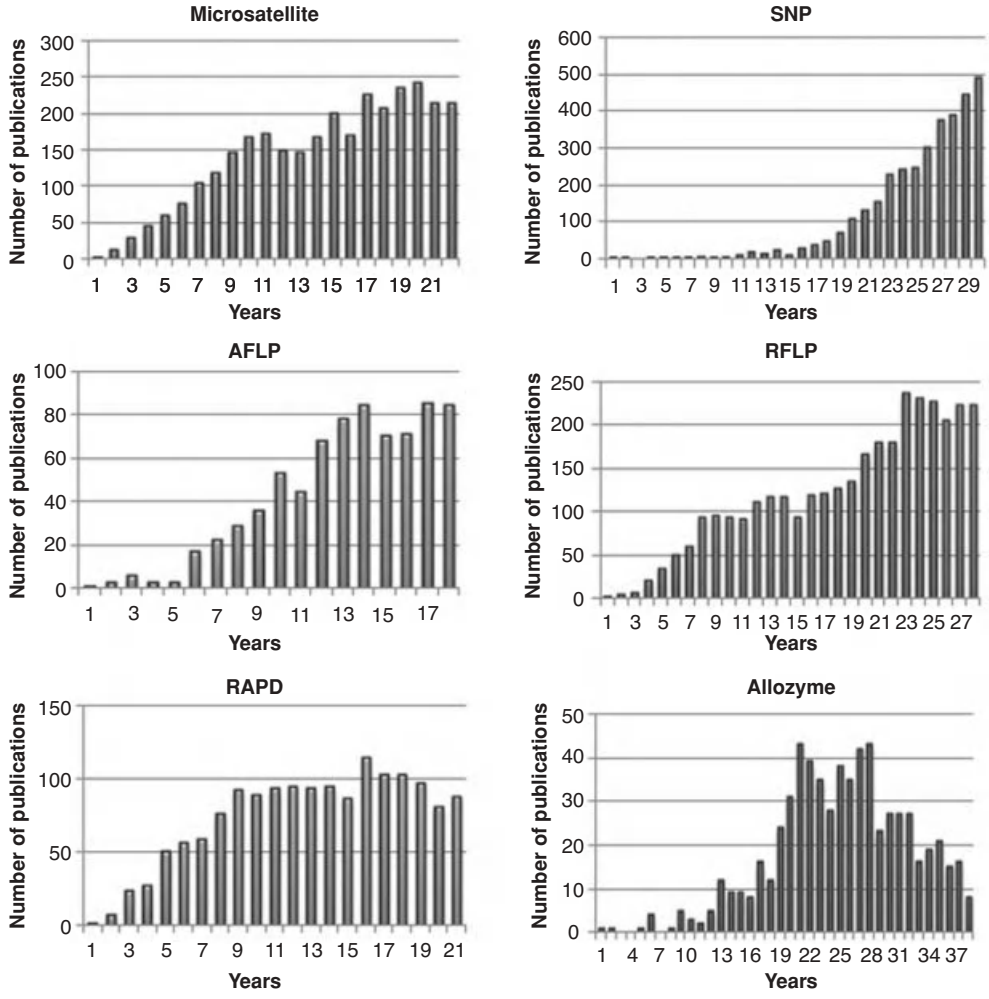

Figure 2.1 Publications generated using various types of markers. The data were obtained by searching the marker name against ScienceDirect database. The Literature searches start from Allozyme (1973), RFLP (1983), RAPD (1990), AFLP (1993), microsatellites (1989), SNP (1981). The *X*-axis shows years after the beginning; numbers on the *Y*-axis are numbers of publications.

developed for catfish (Liu et al., 1998, 1999a; He et al., 2003; Wang et al., 2008, 2010a; Liu, 2011a, 2011b), Atlantic salmon (e.g., Moen et al., 2008a; Hayes et al., 2007; Dominik et al., 2010), Atlantic cod (Moen et al., 2008b, 2009), Japanese flounder (Nakayama et al., 2007), common carp (Kongchum et al., 2010), etc. In aquaculture studies to date, DNA markers are primarily applied for individual identification, parentage assignment, population genetics, genetic mapping, sex identification and marker assistant selection (MAS). Among all these applications, genetic linkage map is one of the powerful tools for functional genomics analysis. Genetic linkage maps have been developed for many aquaculture species such as Nile tilapia (Kocher et al., 1998; Agresti et al., 2000; Lee et al., 2005a, 2011), Atlantic salmon (Moen et al., 2004, 2008a; Gilbey et al., 2004; Phillips et al., 2009; Lorenz et al., 2010), channel catfish (Waldbieser et al., 2001; Liu et al., 2003; Li et al., 2007b; Kucuktas et al., 2009), rainbow

trout (Young et al., 1998; Nichols et al., 2003; Rexroad et al., 2008), Arctic charr (Woram et al., 2004), Pacific oyster (Li and Guo, 2004), eastern oyster (Yu and Guo, 2003; Li and Guo, 2004; Guo et al., 2011), striped bass (Liu et al., 2011), European sea bass (Christiakov et al., 2005), brown trout (Gharbi et al., 2006), gilthead sea bream (Franch et al., 2006), Atlantic halibut (Reid et al., 2007), European flat oyster (Lallias et al., 2007), bighead carp (Liao et al., 2007), silver carp (Liao et al., 2007), coho salmon (McClelland and Naish, 2008), turbot (Ruan et al., 2010) and Japanese eel (Nomura et al., 2011; Unuma et al., 2011), and other fish and shellfish species.

Expressed Sequence Tags (ESTs)

ESTs are powerful tools in gene discovery and transcriptome studies. Also, EST analysis is applied to SNP and microsatellite identification, alternative splicing, and duplication analysis and microarray development. EST resources were rapidly developed in a large number of species. To meet this need, the National Center for Biotechnology Information (NCBI) developed the database dbEST to store EST information. In addition to the raw EST information, some other databases provide EST consensus for further gene analysis, such as the Gene Index database (http://compbio.dfci.harvard.edu/tgi/). There are many species-specific EST database, such as Diatom EST database (http://avesthagen.sznbowler.com/) and Plant Genome database (http://www.plantgdb.org/). Here, we list some important EST databases, which also include ESTs from aquaculture species.

NCBI's Public dbEST

dbEST (http://www.ncbi.nlm.nih.gov/dbEST/), is the largest, publicly available database for EST information. As of early October 2011, there were almost 71 million (70,937,429) ESTs from 1770 different species (dbEST release 100111). Table 2.2 presents the summary of 184 aquaculture and related model fish or shellfish species with EST numbers above 100 from dbEST (Table 2.2). These species include fish, squid, crab, shrimp, shellfish, sea horse, sea hare, starfish, sea cucumber, sea anemone, sea urchin, etc.

NCBI's Public UniGene

Unigene database (http://www.ncbi.nlm.nih.gov/unigene) is constructed to provide a unique set of transcript sequences that appear to come from the same transcription locus (gene or expressed pseudogene), together with information on protein similarities, gene expression, cDNA clone reagents, and genomic location. This database was designed to resolve, or otherwise to reduce, ESTs redundancy problems because ESTs are single pass sequences from either normalized or non-normalized cDNA libraries. Particularly in non-normalized libraries, redundancy can be over thousands for highly abundantly expressed genes. UniGene sorts ESTs into a nonredundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene (Boguski and Schuler, 1995). For aquaculture species, this database includes 12 Actinopterygii species, Florida lancelet, sea lamprey, two Echinoidea species, Pacific white shrimp, California sea hare, owl limpet, and nematode. Apparently, only those with good transcriptome resources are represented in this database.

The Gene Indices

DFCI Gene Indices (formerly, TIGR Gene Indices; Lee and Quackenbush, 2003; Lee et al., 2005b; Antonescu et al., 2010) (http://compbio.dfci.harvard.edu/tgi/) are a collection of 77 species-specific databases that assemble the ESTs and the Expressed Transcripts (ETs) into tentative consensus (TC) sequences. The TC sequence can represent putative gene with

Table 2.2 Existing EST resources for 183 species of aquaculture and related model fish species with 100 or more ESTs.

Species	Common name	Number of ESTs
Danio rerio	Zebrafish	1,488,275
Oryzias latipes	Japanese medaka	666,891
Salmo salar	Atlantic salmon	498,212
Ictalurus punctatus	Channel catfish	354,488
Branchiostoma floridae	Florida lancelet	334,502
Oncorhynchus mykiss	Rainbow trout	287,967
Gasterosteus aculeatus	Three spined stickleback	276,992
Pimephales promelas	Fathead minnow	258,504
Aplysia californica	California sea hare	255,605
Lottia gigantea	Owl limpet	252,091
Gadus morhua	Atlantic cod	229,094
Crassostrea gigas	Pacific oyster	206,388
Litopenaeus vannamei	Whiteleg shrimp	161,241
Strongylocentrotus purpuratus	Purple urchin	141,833
Paracentrotus lividus	Common sea urchin	140,897
Ictalurus furcatus	Blue catfish	139,475
Petromyzon marinus	Sea lamprey	120,731
Oreochromis niloticus	Nile tilapia	119,531
Petrolisthes cinctipes	Flat porcelain crab	97,806
Sparus aurata	Gilthead sea bream	74,877
Fundulus heteroclitus	Atlantic killifish	74,755
Patiria pectinifera	Starfish	56,831
Dicentrarchus labrax	European seabass	55,835
Acropora palmata	Elkhorn coral	43,150
Mytilus californianus	California mussel	42,354
Anemonia viridis	Mediterranean snakelocks sea anemone	39,939
Penaeus monodon	Giant tiger prawn	39,397
Dissostichus mawsoni	Antarctic cod	37,104
Osmerus mordax	Rainbow smelt	36,788
Euprymna scolopes	Hawaiian bobtail squid	35,420
Cyprinus carpio	Common carp	34,316
Anoplopoma fimbria	Sablefish	34,080
Esox lucius	Northern pike	32,833
Squalus acanthias	Spiny dogfish	32,562
Leucoraja erinacea	Little skate	31,167
Homarus americanus	American lobster	29,957
Metridium senile	Brown sea anemone	29,412
Eptatretus burgeri	Inshore hagfish	27,909
Takifugu rubripes	Japanese pufferfish	26,069
Sebastes caurinus	Copper rockfish	23,668
Misgurnus anguillicaudatus	Oriental weatherfish	22,158
Perca flavescens	Yellow perch	21,968
Paralabidochromis chilotes	Lake victoria cichlid	21,652
Hippoglossus hippoglossus	Atlantic halibut	20,836
Mytilus galloprovincialis	Mediterranean mussel	19,617

(*Continued*)

Table 2.2 (*Continued*)

Species	Common name	Number of ESTs
Rutilus rutilus	Common roach	18,470
Eriocheir sinensis	Chinese mitten crab	16,987
Poecilia reticulata	Guppy	16,217
Carcinus maenas	Common shore crab	15,558
Acropora millepora	Staghorn coral	15,389
Paralichthys olivaceus	Japanese flounder	15,234
Crassostrea virginica	Eastern oyster	14,560
Oncorhynchus tshawytscha	King salmon	14,173
Psetta maxima	Turbot	12,427
Lates calcarifer	Barramundi	11,530
Aplysia kurodai	Kuroda's sea hare	11,445
Oncorhynchus nerka	Sockeye salmon	11,389
Sebastes rastrelliger	Grass rockfish	11,207
Thymallus thymallus	Grayling	10,975
Solea senegalensis	Senegalese sole	10,631
Callinectes sapidus	Blue crab	10,563
Fenneropenaeus chinensis	Fleshy prawn	10,446
Astatotilapia burtoni	Burton's mouthbrooder	10,312
Coregonus clupeaformis	Lake whitefish	10,312
Torpedo californica	Pacific electric ray	10,185
Thunnus thynnus	Atlantic bluefin tuna	10,163
Hyriopsis cumingii	Triangle shell mussel	10,156
Cynoglossus semilaevis	Tongue sole	10,128
Salvelinus fontinalis	Brook trout	10,047
Ilyanassa obsoleta	Mud snail	9,639
Portunus trituberculatus	Swimming crab	9,552
Boreogadus saida	Polar cod	9,499
Idiosepius paradoxus	Northern pygmy squid	9,079
Mizuhopecten yessoensis	Japanese scallop	8,987
Carassius auratus	Gold crucian carp	8,953
Lethenteron japonicum	Japanese lamprey	8,788
Xiphophorus maculatus x Xiphophorus helleri	Southern platyfish x green swordtail	8,491
Platichthys flesus	European flounder	8,396
Haliotis asinina	Donkey's ear abalone	8,335
Lithognathus mormyrus	Striped seabream	8,109
Gobiocypris rarus	Rare gudgeon	8,102
Cyprinodon variegatus	Sheepshead minnow	8,099
Apostichopus japonicus	Sea cucumber	7,669
Pinctada martensi	Chinese pearl shell	7,130
Pinctada maxima	Silver-lipped pearl oyster	7,099
Haliotis diversicolor	Japanese abalone	7,088
Argopecten irradians	Bay scallop	7,057
Euphausia superba	Antarctic krill	6,142
Haliotis discus hannai	Japanese disc abalone	5,787
Venerupis philippinarum	Japanese carpet shell	5,656
Holothuria glaberrima	Brown rock sea cucumber	5,615

Table 2.2 (*Continued*)

Species	Common name	Number of ESTs
Sinonovacula constricta	Razor clam	5,296
Siniperca chuatsi	Chinese perch	5,223
Mytilus edulis	Blue mussel	5,036
Oncorhynchus kisutch	Coho salmon	4,942
Ctenopharyngodon idellus	Grass carp	4,732
Venerupis decussatus	Clam	4,645
Miichthys miiuy	Mi-iuy croaker	4,569
Seriola quinqueradiata	Five-ray yellowtail	3,889
Celuca pugilator	Atlantic sand fiddler crab	3,646
Pagrus major	Red sea bream	3,488
Dreissena rostriformis bugensis	Quagga mussel	3,429
Acipenser sinensis	Chinese sturgeon	3,384
Chlamys farreri	Chinese scallop	3,335
Macrobrachium nipponense	Oriental river shrimp	3,256
Marsupenaeus japonicus	Kuruma prawn	3,156
Harpagifer antarcticus	Antarctic spiny plunderfish	3,138
Poeciliopsis turneri	Blackspotted livebearer	2,735
Acipenser transmontanus	White sturgeon	2,704
Panulirus japonicus	Japanese spiny lobster	2,673
Hemicentrotus pulcherrimus	Sea urchin	2,469
Perca fluviatilis	European perch	2,226
Chiloscyllium plagiosum	Whitespotted bambooshark	2,120
Gecarcoidea natalis	Christmas island red crab	2,118
Macrobrachium rosenbergii	Giant fresh water prawn	2,116
Meretrix meretrix	Asiatic hard clam	2,111
Chiloscyllium griseum	Grey bambooshark	2,051
Carassius auratus langsdorfii	Japanese silver crucian carp	2,005
Sebastes schlegelii	Korean rockfish	1,980
Clarias batrachus	Walking catfish	1,937
Zosterisessor ophiocephalus	Grass goby	1,749
Epinephelus coioides	Orange-spotted grouper	1,694
Labeo rohita	Indian major carp	1,631
Scyliorhinus canicula	Small-spotted catshark	1,600
Pseudopleuronectes americanus	Winter flounder	1,483
Haliotis discus	Abalone	1,393
Pinctada fucata	Pearl oyster	1,335
Poeciliopsis presidionis	Sinaloa livebearer	1,301
Larimichthys crocea	Croceine croaker	1,258
Patiria miniata	Bat star	1,249
Sarotherodon melanotheron	Blackchin tilapia	1,172
Cancer magister	Dungeness crab	1,137
Protopterus aethiopicus	Marbled lungfish	1,133
Litopenaeus setiferus	White shrimp	1,042
Nautilus pompilius	Chambered nautilus	1,037
Dreissena polymorpha	Zebra mussel	998
Pseudocentrotus depressus	Sea urchin	941

(*Continued*)

Table 2.2 (*Continued*)

Species	Common name	Number of ESTs
Monopterus albus	Swamp eel	904
Sepiella maindroni	Cuttlefish	899
Plecoglossus altivelis altivelis	Ayu	898
X. maculatus	Southern platyfish	847
Haliotis discus discus	Disc abalone	839
Pacifastacus leniusculus	Signal crayfish	802
Thalassophryne nattereri	Niquim	775
Harpagifer bispinis	Magellan plunderfish	765
Mytilus coruscus	Sea mussel	719
Fenneropenaeus indicus	Indian white prawn	714
C. idella	Grass carp	640
Opsanus beta	Gulf toadfish	619
Paralichthys lethostigma	Southern flounder	596
Carcinoscorpius rotundicauda	Southeast asian horseshoe crab	575
Argopecten purpuratus	Chilean northern scallop	565
Verasper variegatus	Spotted flounder	524
Branchiostoma belcheri	Amphioxus	506
Ilyoplax pusilla	Dotillid crab	438
Litopenaeus stylirostris	Pacific blue shrimp	416
Coelomactra antiquata	Xishi tongue Clam	406
Clupea harengus	European pilchard	397
Dicathais orbita	Cart-rut shell	386
Sebastes inermis	Darkbanded rockfish	381
Solaster stimpsonii	Striped sun sea star	371
Oreochromis mossambicus	Mozambique tilapia	355
Gandalfus yunohana	Hydrothermal vent crab	310
Haliotis diversicolor supertexta	Taiwan abalone	306
Scomber japonicus	Chub mackerel	304
Muraenesox cinereus	Daggertooth pike conger	296
Protopterus annectens	African lungfish	261
Haliotis tuberculata	Green ormer	222
Anguilla japonica	Japanese eel	196
Saccostrea glomerata	Sydney rock oyster	183
Pelodiscus sinensis	Chinese softshell turtle	178
Acropora tenuis	Purple tipped acropora	176
Brienomyrus brachyistius	Electric fish	153
Heliocidaris erythrogramma	Sea urchin	151
Acropora microphthalma	Antler coral	142
Hippocampus comes	Tiger tail seahorse	139
Mercenaria mercenaria	Florida stone crab	137
Neocaridina denticulata	Cherry shrimp	132
Sebastiscus marmoratus	False kelpfish	126
Anguilla anguilla	European freshwater eel	121
Cherax quadricarinatus	Australian red claw crayfish	120
Pomatomus saltatrix	Bluefish	116
Pampus argenteus	Silver pomfret	105

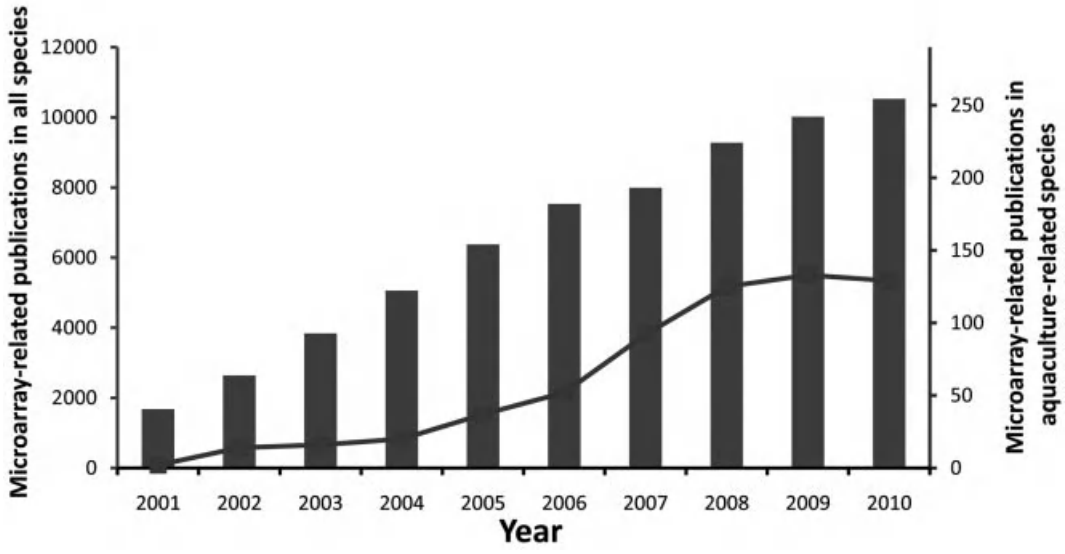

Figure 2.2 Publications of microarray-related work in all fields and in aquaculture-related species.

functional annotation (Lee et al., 2005b). The 77 species include 29 animals, 25 plants, 8 fungi, and 15 protists. Among them, there are 12 fish species.

Public Sigenae Contig Browser

Sigenae EST Contig Browser (http://public-contigbrowser.sigenae.org:9090/index.html) is based on Ensembl software platform and provides simple and rapid visual access to information related to assembly and similarity feature of EST contigs. Five aquaculture species are included in this system, which are European seabass, rainbow trout, Pacific oyster, gilthead sea bream, and salmon.

The US National Animal Genome Project Bioinformatics Database

The USDA National Animal Genome Project is also referred to as NRSP-8 project. The Bioinformatics Coordinators of this project hosts a series of databases related to agricultural animals including aquaculture species, but currently the representation of aquaculture species is relatively small. The database can be accessed through http://www.genome.iastate.edu/bioinfo/.

Microarrays

Gene expression profiling with microarray is one of the most widely used technologies within functional genomics. Comparing to the traditional single or several gene identification method, microarray allows the global identification of DNA samples and examination of gene expression in individual cells or tissues under different conditions simultaneously. The core principle of microarray is hybridization between two DNA strands: probe (feature), which is attached on the surface of chip, and target, which is from the interested sample with fluorescence labeling.

In recent years, microarray technology has advanced significantly, including advances in slides, printing devices, scanners and analyzing softwares. Figure 2.2 shows the various microarray-related publications in the past ten years, which reflected the rapidly increased applications of microarray technology. With the availability of more and more

genetic resources such as ESTs, transcriptome sequences, and whole genome sequences, microarray technologies have been dramatically advanced and broadly applied in aquaculture and related model fish species. For instance, zebrafish, which served as a model for the application of microarray technologies, with a large amount of available resources, have well utilized microarray technologies for various purposes (Ton et al., 2002, 2003; Lo et al., 2003; Mathavan et al., 2005). Several generations of arrays from salmonid species were generated and integrated into salmonid research. To date, the largest salmonid microarray, which was recently developed, consists of 44K oligo features mainly based on Atlantic salmon and rainbow trout EST database (Jantzen et al., 2011). Peatman et al. (2007, 2008) constructed a total 28K oligo microarray to study the gene expression profiling in catfish after bacterial infection. Table 2.3 summarizes additional information regarding microarray platforms utilized in major aquaculture species. More details are accessible in Gene Expression Omnibus (GEO) in NCBI (http://www.ncbi.nlm.nih.gov/geo).

The key goal of microarray application in aquaculture is to screen gene expression profile temporally or spatially, and identify genes potentially involved in production and performance traits such as disease resistance (Rise et al., 2004; Meijer et al., 2005; Darawiroj et al., 2008; Peatman et al., 2007; Park et al., 2009; Ramírez-Gómez et al., 2009; De Zoysa et al., 2011; Aoki et al., 2011), growth rate (Ton et al., 2002; Rise et al., 2006), response to environmental stress (Ton et al., 2003; Kalujnaia et al., 2007; Buckley and Somero, 2009; Boswell et al., 2009; Calduch-Giner et al., 2010) and reproduction (Karoonuthaisiri et al., 2009). Because of insufficient funds and relatively small research community, the genetic and genomic resources for most aquaculture species are limited, which resulted in the limited availability of microarray analysis. It is possible to utilize the heterogeneous microarray hybridization, which applies microarray developed from one species to another related species. In this regard, the Atlantic salmon microarrays developed from the GRASP and cGRASP projects are perhaps among the most utilized microarrays in aquaculture species, whether for homogeneous within-species studies, or heterogeneous applications for across-species studies. We will not provide a summary of these detailed studies, but rather just provide some examples among various aquaculture species. For instance, Renn et al. (2004) constructed a cDNA microarray from the African cichid fish and utilized it for the gene expression profiling across other eight different fish species, which demonstrated that heterogeneous microarray hybridization can generate biologically meaningful data even in relatively distantly related species. The first utilization of transcriptomic approach in European sea bass was achieved by using a microarray platform containing 9K rainbow trout cDNA clones (Darias et al., 2008). A 16K salmonid cDNA microarray was used to compare the patterns of gene transcription between dwarf and normal whitefish (St-Cyr et al., 2008). Once again, the 16K and the 44K microarray platforms that generated GRASP and cGRASP have been widely used to address various types of biological questions such as thermo-tolerance related candidate genes, interspecific hybridization, expression profiles of transgenic fish, and diseases resistance-related genes (Rise et al., 2004, 2006; von Schalburg et al., 2005). Along with comparative genomic approaches, such studies have allowed the identification of candidate genes for performance and production traits (Li et al., 2011; Quinn et al., 2011). The currently developed high-throughput next-generation sequencing technologies seem to have the potential to significantly supplement microarray technology. However, microarray is still a powerful tool for routine studies of selected target sequences. Moreover, the next-generation sequencing can help the application of microarray in aquaculture species overcome the genetic-resource limitation, by providing more available sequences. More efforts are needed to adapt and exploit the advances of the applications of microarray in aquaculture.

Table 2.3 A summary of the currently available microarray platforms with aquaculture and related model fish species.

Species	Common name	Array type	Number of features	Probe size	Company	References
Anguilla anguilla	European eel	cDNA	6,144 in triplicate	N/A	Liverpool Microarray Facility	Kalujnaia et al., 2007
Astatotilapia burtoni	African cichlid fish	cDNA	4,500	N/A	GeneMachines	Renn et al., 2004
Carcinus maenas	Green crab	Oligo	4,462	50-mer	Parallel Synthesis Technologies Inc.	Towle et al., 2011
Crassostrea spp.	Oyster	cDNA	27,496	N/A	Genetix	Jenny et al., 2007
Crassostrea spp.	Oyster	Oligo	12,000	60-mer	NimbleGen	Wang et al., 2010b
Crassostrea gigas	Pacific oyster	cDNA	11,088	N/A	MPI Molecular Genetics	Fleury et al., 2010
Cyprinus carpio	Common carp	cDNA	26,202	N/A	ArrayJet	Williams et al., 2008
Danio rerio	Zebrafish	cDNA	4,512	N/A	Affymetrix	Ton et al., 2002
D. rerio	Zebrafish	cDNA	11,480	N/A	Sigma	Lo et al., 2003
D. rerio	Zebrafish	Oligo	16,416	65-mer	Custom-built DNA microarrayer	Mathavan et al., 2005
D. rerio	Zebrafish	cDNA	5,184	N/A	Genetic Microsystems	Handley-Goldstone et al., 2005
Dicentrarchus labrax	European sea bass	Oligo	40,000	60-mer	Agilent SurePrint™ technology	Ferraresso et al., 2010
Dreissena polymorpha	Zebra mussle	cDNA	716 in triplicate	N/A	Genomic Solutions	Xu and Faisal, 2009
Epinephelus coioides	Orange-spotted grouper	cDNA	413 in duplicate	N/A	GE Healthcare	Xu et al., 2010
Euphausia superba	Antarctic krill	cDNA	10,176	N/A	Genetix	Seear et al., 2010.
Fenneropenaeus chinensis	Chinese shrimp	cDNA	3,136	N/A	Biostar Gene Chip	Wang et al., 2006
Fugu rubripes	Fugu	cDNA	1,842 in duplicate	1 kb	HGMP	Hogstrand et al., 2002
Fundulus heteroclitus	Atlantic killifish	cDNA	1,149 genes	N/A	In-house designed	Oleksiak et al., 2002
Gadus morhua	Atlantic cod	Oligo	41,472	50-mer	ACRI	Booman et al., 2011
G. morhua	Atlantic cod	cDNA	16,348	N/A	Genomic Solutions®	Edvardsen et al., 2011
Gasterosteus aculeatus	Three-spined stickleback	cDNA	9,692	N/A	Birmingham Functional Genomics Laboratory	Geoghegan et al., 2008
Gillichthys mirabilis	Goby	cDNA	12,661	N/A	Telechem	Gracey, 2008
Haliotis asinina	Vetigastropod	cDNA	5,541 in duplicate	N/A	Genetic Microsystems	Williams et al., 2009
Haliotis discus discus	Disk abalone	cDNA	4.2 k	N/A	Genomictree Inc.	De Zoysa et al., 2011
Haliotis diversicolor	Small abalone	cDNA	3,590	N/A	Cartesian Technologies	Jia et al., 2011
Harpagifer antarcticus	Antarctic plunderfish	cDNA	10,371 in duplicate	N/A	Genetix	Thorne et al., 2010
Hippoglossus hippoglossus	Atlantic halibut	Oligo	39 936	50-mer	Atlantic Microarray Facility	Douglas et al., 2008

(*Continued*)

Table 2.3 (*Continued*)

Species	Common name	Array type	Number of features	Probe size	Company	References
Holothuria glaberrima	Sea Cucumber	Oligo	8 × 15K	60-mer	Agilent	Ramírez-Gómez et al., 2009
Ictalurus sp.	Catfish	Oligo	19,000	24-mer	NimbleGen	Li and Waldbieser, 2006
Ictalurus sp.	Catfish	Oligo	28,000	24-mer	Nimblegen	Peatman et al., 2007
Litopenaeus vannamei	Pacific whiteleg shrimp	cDNA	2,469 in duplicate	N/A	QArrayMax	Robalino et al., 2007
Micropterus salmoides	Largemouth bass	Oligo	45,220	60-mer	Agilent	Garcia-Reyero et al., 2008
Mytilus galloprovincialis	Mediterranean mussel	cDNA	1,758 in duplicate	N/A	ArrayIt	Venier et al., 2006
Oryzias latipes	Japanese Medaka	Oligo	26,689	60-mer	NimbleGen	Kishi et al., 2008
Paralichthys olivaceus	Japanese flounder	cDNA	871 clones	N/A	DNA Chip Research Inc.	Kurobe et al., 2005
Penaeus monodon	Black tiger shrimp	cDNA	3,853 in duplicate	N/A	Genetic Microsystems	de la Vega et al., 2007
P. monodon	Black tiger shrimp	cDNA	1,026 in duplicate	N/A	DNA Chip Research Inc	Wongpanya et al., 2007
P. monodon	Black tiger shrimp	cDNA	4,992	N/A	In-house designed	Karoonuthaisiri et al., 2009
Petrolisthes cinctipes	Porcelain crab	cDNA	13,824	N/A	UCSF's Genomics and Proteomics Core Facility	Teranishi and Stillman, 2007
Pimephales promelas	Fathead minnow	Oligo	2,000 in duplicate	60-mer	Agilent	Larkin et al., 2007
Platichthys flesus	European flounder	cDNA	27,648	N/A	Birmingham Functional Genomics Laboratory	Diab et al., 2008
Portunus trituberculatus	Swimming crab	cDNA	2,426 in triplicate	N/A	CapitalBio Corporation	Xu and Liu, 2011
Psetta maxima	Turbot	cDNA	1,920 in duplicate	N/A	Biotechnology Research Institute	Park et al., 2009
P. maxima	Turbot	Oligo	8 × 15K	60-mer	Agilent	Millán et al., 2010
Ruditapes philippinarum	Manila clam	Oligo	40,332	60-mer	Agilent	Milan et al., 2011
Salmonid	Salmonidae	cDNA	16,950	N/A	Genomic solutions	von Schalburg et al., 2005
Salmonid	Salmonidae	cDNA	32,000	N/A	Genetix	Koop et al., 2008
Salmonid	Salmonidae	Oligo	44,000	60-mer	Agilent	Jantzen et al., 2011
Scylla paramamosain	Mud crab	cDNA	5,160	N/A	Jimei University	Zou et al., 2011
Seriola quinqueradiata	Yellowtail	cDNA	1,001 in duplicate	>200 bp	DNA Chip Research Inc.	Darawiroj et al., 2008
Solea senegalensis	Senegalese sole	Oligo	5,087 unique genes	50/60-mer	Agilent	Cerdà et al., 2008
Sparus aurata	Gilthead sea bream	Oligo	10,176	60-mer	Agilent	Sarropoulou et al., 2005
S. aurata	Gilthead sea bream	Oligo	39,379	60-mer	Agilent SurePrint™ technology	Ferraresso et al., 2008
S. aurata	Gilthead sea bream	cDNA	18,490 in duplicate	N/A	Max Planck Institute facilities	Calduch-Giner et al., 2010

Sequence Read Archive (SRA)

The combination of significantly lower cost and increased speed and throughput of sequencing gives rise to an explosive growth of data submitted into the primary next-generation sequence data archive, the Sequence Read Archive (SRA). The preservation of experimental data is a crucial part of the scientific record, and increasing numbers of journals and funding agencies require the submission of the next-generation sequence data. All these events promoted the emergence of the SRA.

In response to the research community's need as a public and global repository for the next-generation sequence data storage and retrieval, the SRA was first established. It is operated under the guidance of the International Nucleotide Sequence Database Collaboration (INSDC). INSDC has three partners: they are the National Center for Biotechnology Information (NCBI), the European Bioinformatics Institute (EBI), and the DNA Data Bank of Japan (DDBJ). The SRA is accessible at http://www.ncbi.nlm.nih.gov/Traces/sra from NCBI, at http://www.ebi.ac.uk/ena from EBI and at http://trace.ddbj.nig.ac.jp from DDBJ (Leinonen et al., 2011a).

Raw sequencing data from the next-generation sequencing platforms such as Roche 454 GS System®, Illumina Genome Analyzer®, Applied Biosystems SOLiD® System, Helicos Heliscope®, Complete Genomics®, and Pacific Biosciences SMRT® can be stored in SRA (http://www.ncbi.nlm.nih.gov/sra). The data are organized into four types of records including studies (SRP accessions), experiments (SRX accessions), samples (SRS accessions), and runs (SRR accessions). Each study could contain one or more experiments and each experiment could contain one or more runs. Owing to the high throughput character of the next-generation sequencing, every single run could contain millions of individual reads (Sayers et al., 2011). In SRA, Illumina data currently occupies about three-fourths of the repository, while the other platforms share the rest one-fourth (http://www.bio-itworld.com/NGS-NCBI.html). As a basic platform to support large-scale studies or as a complement to publications, SRA service provides route for data dissemination. It also provides data access to users for re-analysis of existing data and to review the descriptions of studies and experiments independently of experimental data (http://www.ebi.ac.uk/industry/Documents/workshop-materials/newsequence291009/The_Sequence_Read_Archive-Guy_Cochrane.pdf.).

The European Nucleotide Archive (ENA) is another primary nucleotide-sequence archive that captures and presents information related to experimental workflows. In order to support and promote the use of nucleotide sequencing as an experimental research platform by providing data submission, archive, and search, as well as the download services (Leinonen et al., 2011b), ENA was established. The data recorded in ENA includes three types of information: the input information, output machine data, and interpreted information (http://www.ebi.ac.uk/ena/).

DDBJ Sequence Read Archive (DRA) provides all public SRA data generated by next-generation sequencing machines. It is archiving the data in a close collaboration with SRA in NCBI and ERA (European Reads Archive) in EBI (http://www.ddbj.nig.ac.jp/intro-e.html#insdc).

Applications

SRA plays an important role in functional genomics studies since it stores data for more than 7000 studies to date for a huge variety of experimental purposes, such as gene expression analysis, whole genome sequencing, genetic marker identification, comparative transcriptomic studies, zinc finger nuclease studies, epigenetic profiling, examination of small RNAs, and other purposes. Over 100 studies have been conducted using next-generation sequencing for various studies such

as gene expression patterns, transcriptome analyses, SNP identification involving aquaculture species, such as Atlantic salmon, catfish, rainbow trout, guppy, striped bass, oysters, scallops, and sea urchin, and related model fish species such as zebrafish and medaka. Details for SRA applied in aquaculture species and related model fish species are listed in Table 2.4.

SNP Genotyping Platforms

SNP has been considered as one of the most powerful markers because of its properties of high abundance (one SNP in every 1250 bp in human genome), adaption to automation, and high resolution to reveal hidden polymorphisms which other markers cannot detect (Liu and Cordes, 2004). As a result of this importance, SNP discovery has become a hot spot for molecular marker development. With the development of genetic and genomic research in aquaculture species, SNP genotyping is playing an increasingly important role in high-density linkage mapping, quantitative trait loci (QTL) analysis, fine mapping, and whole genome-based selection (Liu, 2007, 2011a, 2011b).

Historically, SNPs have been given great attention even in early period of genome research, although methods applied in the last 20 years were mostly small scale. These early methods included denaturing gradient gel electrophoresis (DGGE) (Cariello et al., 1988), single strand conformational polymorphism assay (SSCP) (Suzuki et al., 1990), ligation chain reaction (LCR, Kalin et al., 1992), and allele specific oligonucleotide (ASO, Malmgren et al., 1996). Recently, various strategies have been utilized for the discovery of SNPs including direct sequencing, data mining from ESTs, data mining from the overlapping BAC end sequences, and SNP identification from Reduced Representation Shotgun sequencing (RRS)(e.g., Van Tassell et al., 2008).

More recently, a few platforms are available for genotyping SNPs in different projects. Among them, MassArray (Sequenom), SNPstream (Beckman Coulter) and SnaPshot Multiplex System (Applied Biosystems) are based on the single-base extension; iSelct HD Custom (Illumina) and GoldenGate (Illumina) use allele-specific primer extension; TaqMan OpenArray (Applid Biosystems) and Dynamic Array (Fluidigm) are designed from the TaqMan technology; MyGeneChip Custom Arrays (Affymetrix) use the chemistry of differential hybridization. Affymetrix has adopted the Axiom SNP platform. The Axiom Genotyping Solution includes predesigned and customized array plates with validated genomic content from the Axiom Genomic Database as well as complete reagent kits, data analysis tools, and a fully automated workflow utilizing the GeneTitan® Multi-Channel (MC) Instrument (http://www.affymetrix.com/browse/staticHtmlContentTemplate.jsp?staticHtmlMediaId=m1361217&isHtmlStatic=true&navMode=35810&aId=productsNav). With Axiom, hundreds of thousands or millions of features can be custom-designed for the species of interest. It is anticipated that in the near future, such systems will be applied to aquaculture species.

In addition to the platforms mentioned above, RAD (restriction-site associated DNA) markers sequencing (Miller et al., 2007a, 2007b) is an integrated platform for SNP discovery and genotyping. Using RAD platform, Nathan A. Baird et al. (2008) identified more than 13,000 SNPs, and mapped three traits in two model organisms, with just less than half the capacity of one Illumina sequencing run. Hohenlohe et al. (2011) used RAD sequencing for the discovery of SNPs to study interspecific hybridization of rainbow trout and westslope cutthroat trout. Table 2.5 outlines the application of primary strategies and SNPs genotyping platforms in aquaculture projects.

Databases for Aquaculture Species

With the rapid progress made in aquaculture species, bioinformatic databases have been constructed to present the comprehensive

Table 2.4 A summary of SRA data available from some fish, shellfish, and aquaculture species. Data obtained from SRA browse as of 09/09/2011. Only economically important or model fish species were listed.

Accession	Species	Common name	Experimental goals	Data	Submitted by
SRP002880	*Apostichopus japonicus*	Sea cucumber	Gene expression during regeneration	LS454-1 run, 175,839 spots, 91M bases	Meercas, 2010
SRP005930	*Acipenser oxyrinchus desotoi*	Gulf sturgeon	Isolation of microsatellite loci	LS454-1run, 82,952M spots, 39M bases	University of Florida, 2011
SRP001144	*Amphilophus astorquii* and *A. zaliosus*	Cichlid fishes	To compare the EST sequence divergence	LS454-2 runs, 563,104 spots, 151.7M bases	University of Konstanz, 2009
SRP004507	*Anguilla Anguilla*	European eel	Identification of genetic markers	LS454-1 run, 310079 spots, 82.6M bases	University of Padua, 2010
SRP004787	*Astatotilapiaburtoni*	African cichlid	Whole genome sequencing	Illumina-20 run, 2.2Gspots, 358.3G bases	BI, 2011
SRP007198	*A. burtoni* and *Ophtalmotilapia ventralis*	African Cichlid Fishes	Comparative Transcriptomics	LS454-2 runs, 1.3M spots, 678.6M bases	Unibas, 2011
SRP000398	*Callorhinchus milii*	Elephant shark	Whole genome sequencing	LS454-2 runs, 914,986 spots, 244.9M bases	MPIMG, 2008
SRP002782	*Clupea pallasii*	Pacific herring	Genetic marker identification	LS454-2 runs, 1.2M spots, 661.6M bases	University of Washington, 2010
SRP003484	*Coregonus clupeaformis, Salmonidae*	Lake whitefish	BAC library construction, screening and clone sequencing	LS454-5 runs, 178004 spots, 92.2M bases	Université Laval, 2010
SRP001171	*Coregonus* spp. *Salmonidae*	Whitefish	SNP study	LS454-6 runs, 627,609 spots, 160.3M bases	Université Laval, 2009
ERP000454	*Ctenopharyngodon idella*	Grass carp	Pilot sequencing	Illumina-2 runs, 53.4M spots, 11.5G bases	SC, 2011
ERP000842	*C. idella*	Grass carp	Metagenome sample taken from adult grass carp intestine and associated environments		2011
SRP001067	*Cyprinus carpio*	Mirror Carp	Expressed sequence tags analysis	LS454-5 runs, 242,263 spots, 60.8M bases	CAFS

(*Continued*)

Table 2.4 (*Continued*)

Accession	Species	Common name	Experimental goals	Data	Submitted by
ERP000282	*Danio albolineatus*	Pearl Danio	Danio species sequencing project	Illumina-6 runs, 291.1M spots, 55.5G bases	SC, 2010
ERP000283	*Danio nigrofasciatus*	Spotted Danio	Danio species sequencing project	Illumina-6 runs, 233.9M spots, 44.5G bases	SC, 2010
ERP000110	*Danio rerio*	Zebrafish	For targeting induced local lesions in genomes	Illumina	SC, 2010
ERP000426	*D. rerio*	Zebrafish	For targeting induced local lesions in genomes	Illumina	SC, 2010
ERP000447	*D. rerio*	Zebrafish	Sanger zebrafish sequencing	Illumina-12 runs, 228.6M spots, 35.5G bases	SC, 2010
SRP003361	*D. rerio*	Zebrafish	Zinc finger nucleases study	Illumina-14 runs, 14.8M spots, 531.5M bases	GEO, 2010
ERP000247	*D. rerio*	Zebrafish	Creation of targeted mutations in the zebrafish using zinc finger nucleases	Illumina-2 runs, 11.3M spots, 417.5M bases	SC, 2010
ERP000248	*D. rerio*	Zebrafish	For targeting induced local lesions in genomes	Illumina-2 runs, 12.6M spots, 451.8M bases	SC, 2010
ERP000635	*D. rerio*	Zebrafish	Zebrafish transcriptome during early development	Illumina-2 runs, 315.6M spots, 15.8G bases	KI-BN, 2011
SRP003472	*D. rerio*	Zebrafish	U1C protein in alternative splicing regulation	Illumina-2 runs, 67.3M spots, 5.1G bases	Justus-Liebig University, 2010
SRP002130	*D. rerio*	Zebrafish	Epigenetic profiling	Illumina-21 run, 139.9M spots, 7.9G bases	GEO, 2011
ERP000724	*D. rerio*	Zebrafish	Zebrafish Tilling	Illumina-24 runs, 115.0M spots, 8.5G bases	SC, 2011
ERP000016	*D. rerio*	Zebrafish	Transcriptome from a range of tissues and developmental stages	Illumina-28 runs, 376.3M spots, 41.1G bases	SC, 2009
SRP002159	*D. rerio*	Zebrafish	Traf6 function in the innate immune response	Illumina-4 runs, 60M spots, 3.1G bases	GEO, 2010

SRP001016	*D. rerio*	Zebrafish	Deep sequencing of the zebrafish transcriptome response to mycobacterium infection	Illumina-4 runs, 80.1M spots, 2.9G bases	GEO, 2009
SRP002411	*D. rerio*	Zebrafish	Examination of small RNAs	Illumina-5 runs, 15.1M spots, 540.5M bases	GEO, 2010
ERP000098	*D. rerio*	Zebrafish	To test the viability of Zebrafish mapped clones in nonindexed pools	Illumina-6 runs, 115.7M spots, 12.5G bases	SC, 2010
ERP000191	*D. rerio*	Zebrafish	Zebrafish mapped clones in nonindexed pools	Illumina-61 runs, 1G spots, 154.1G bases	SC, 2010
SRP007331	*D. rerio*	Zebrafish	Tdrd1 function	Illumina-7 runs, 146M spots, 5.5G bases	GEO, 2011
ERP000400	*D. rerio*	Zebrafish	Transcriptome from a range of tissues and developmental stages	Illumina-7 runs, 212.3M spots, 29.5G bases	SC, 2010
ERP000263	*D. rerio*	Zebrafish	Zebrafish gene 3 prime end pull down for genome annotation	Illumina-7 runs, 51.3M spots, 8.9G bases	SC, 2010
ERP000232	*D. rerio*	Zebrafish	Sequence of AB and uebingen strains of zebrafish	Illumina-96 runs, 1.3G spots, 169.8G bases	SC, 2010
SRP000652	*D. rerio*	Zebrafish	Zebrafish IgH sequencing	LS454-14 runs, 2.1M spots, 479.7M bases	Stanford University, 2009
SRP005640	*D. rerio*	Zebrafish	Zebrafish development	LS454-51 run, 14.4M spots, 4.6G bases	Stanford University, 2011
DRP000321	*Eptatretus burgeri*	Inshore hagfish	Transcriptome analysis	LS454-1 run, 157,457 spots, 41.9M bases	RIKEN_CDB, 2011
ERP000097	*Gadus morhua*	Atlantic cod	Whole genome sequencing	LS454-1 200 000 reads	University of Oslo, 2010
SRP001034	*Gasterosteus aculeatus*	Threespine stickleback	Whole genome sequencing	454 and Illumina	
SRP001747	*G. aculeatus*	Threespine stickleback	To identify SNPs	Illumina-8 run, 101.8M spots, 5.1G bases	University of Oregon, 2010

(*Continued*)

Table 2.4 (*Continued*)

Accession	Species	Common name	Experimental goals	Data	Submitted by
SRP004090	*Haliotis midae*	South African abalone	To characterize the transcriptome	Illumina-3 runs, 19.9M spots, 1.1G bases	University of Stelle, 2010
SRP003814	*Ictalurus furcatus* and *I. punctatus*	Blue and channel catfish	Generation of gene-associated SNP for development of SNP array	Illumina-4 runs, 252.9M spots, 43.2G bases	Auburn University, 2010
SRP001718	*Larimichthys crocea*	Yellow croaker	Transcriptome and expression profiling because of Aeromonas hydrophila	Illumina-3 run, 17.4M spots, 1.4G bases	BI, 2010
SRP000436	*Latimeria chalumnae*	West Indian Ocean coelacanth	Deep coverage WGS	Illumina-52 runs, 6.3G spots, 987.5G bases	BI, 2011
SRP000282	*Lepisosteus oculatus*	Spotted Gar	Deep coverage sequencing and assembly of WGS	Illumina-12 runs, 841.2M spots, 176.7G bases	BI, 2008
SRP004473	*L. oculatus*	Spotted Gar	Teleost genome duplication	Illumina-13 run, 374.8M spots, 30G bases	University of Oregon, 2010
SRP001484	*Loricaria gr. cataphracta*	Nonmodel catfish	De novo transcriptome assembly	Illumina-1 run, 10.2M spots, 778.6M bases	University of Geneva, 2009
SRP004788	*Metriaclima zebra*	Tilapia	Whole genome sequencing	Illumina-13 run, 1.1G spots, 203.6G bases	BI, 2011
SRP001052	*Morone saxatilis*	Striped bass	An ovarian transcriptome	LS454-1 run, 230,151 spots, 58.7M bases	SCDNR, 2009
SRP004799	*Neolamprologus brichardi*	Cichlid fish	Whole genome sequencing	Illumina-11 run, 1.6G spots, 278.3G bases	BI, 2010
SRP000194	*Nothobranchius furzeri*	Turquoise killifish	Low redundancy whole genome sequencing	LS454-6 runs, 1.6M spots, 249M bases	IMBGA, 2008
DRP000322	*Oncorhynchusmykiss*	Rainbow trout	To identify genes expressed in fish adipose	LS454-1 run, 33,131 spots, 20M bases	TUMSATGS, 2010
SRP005674	*O. mykiss*	Rainbow trout	To detect differential gene expression	LS454-12 run, 1.5M spots, 846.2M bases	INRA, 2011

SRP001007	*O. mykiss*	Rainbow trout	Transcriptome study	LS454-2 runs, 1.3M spots, 609M bases	West Virginia University, 2009
SRP007371	*O. mykiss*	Rainbow trout	Generation of a reference transcriptome	LS454-2 runs, 3.3M spots, 1.7G bases	National Center for Cool and Cold Water Aquaculture, 2011
SRP004174	*O. mykiss* and *O. clarkii lewisi*	Rainbow trout and westslope cutthroat trout	SNP detection	Illumina-1 run, 27.9M spots, 1.7G bases	University of Oregon, 2010
SRP001084	*Oreochromis niloticus*	Nile Tilapia	BAC clone sequencing	LS454-1 run, 11,696 spots, 3.3M bases	BI, 2009
SRP004172	*O. niloticus*	Nile Tilapia	Whole genome sequencing	LS454-39 runs, 1.8G spots, 293.4G bases	BI, 2011
DRP000221	*Oryzias latipes*	Medaka	Whole genome sequencing	Illumina-1 run, 47.1M spots, 4.8G bases	KEIO-SM, 2010
SRP000304	*O. latipes*	Medaka	Chromatin-associated periodicity study	Illumina-12 runs, 185.9M spots, 6.7G bases	UTGSFSCB-ML, 2008
SRP004363	*O. latipes*	Medaka	Sequencing	Illumina-17 run, 168.3M spots, 6.1G bases	JGI, 2010
SRP002552	*S. purpuratus*	Sea Urchin	Genome and transcriptome sequencing	LS454-5 runs, 2.6M spots, 699.9M bases	BCM, 2010
SRP001081	*Paralabidochromis*	Cichlid	BAC sequencing	LS454-8 runs, 72,901 spots, 20.4M bases	BI, 2009
SRP004922	*Paralichthys olivaceus*	Japanese flounder	Small RNA during metamorphosis	Illumina-1 run, 2.2M spots, 82.5M bases	GEO, 2010
SRP001396	*P. olivaceus*	Cardeau hirame	Expressed sequence tags analysis	LS454-2 runs, 182,851 spots, 45.0M bases	CAFS, 2009
SRP004881	*Patinopecten yessoensis*	Yesso scallop	Transcriptome sequencing	LS454-1 run, 970,422 spots, 433.7M bases	OUC, 2010
DRP000403	*P. yessoensis*	Pygmy squid, nautilus and scallops	Comparative genome structure analyses	LS454-6 runs, 1.3M spots, 788.6M bases	OCHA_AP, 2011

(*Continued*)

Table 2.4 (*Continued*)

Accession	Species	Common name	Experimental goals	Data	Submitted by
SRP003777	*Petromyzon marinus*	Sea lamprey	Whole genome sequencing	Illumina-42 runs, 301M spots, 24.1G bases	WUGSC, 2010
SRP002635	*Pinctada margaritifera*	Pearl oyster	Expressed Sequence Tags library analysis	LS454-1 run, 276,735 spots, 75M bases	Skuldtech, 2010
SRP005402	*Poecilia reticulata*	Guppy	Gene expression differences	454 and Illumina	University of Toronto, 2011
SRP004869	*Pundamilia nyererei*	Cichlid fish	Whole genome sequencing	Illumina-20 run, 1.6G spots, 243.2G bases	BI, 2011
SRP002685	*Ruditapes philippinarum*	Clam	To find genomic regions showing reduced gene flow	LS454-2 runs, 298,628 spots, 80.6M bases	University of Sheffi, 2010
SRP002685	*R. philippinarum*	Manila clam	Global gene expression profiling	LS454-1 run, 457,717 spots, 285.1M bases	University of Padua, 2010
SRP002117	*Salmo salar*	Atlantic salmon	To look for possible infectious agent(s)	LS454-1 run, 469,277 spots, 167.7M bases	National Veterinary, 2010
SRP005188	*Salvelinus fontinalis*	Brook charr	Sequencing a normalized cDNA library	LS454-1 run, 399515 spots, 225.9M bases	Université Laval, 2011
SRP001186	*Salvelinus namaycush*	Lake trout	Phenotypic differentiation study	LS454-2 runs, 478,688 spots, 122.9M bases	University of Wiscon, 2009
DRP000320	*Scyliorhinus torazame*	Cloudy catshark	Transcriptome analysis	LS454-1 run, 162,877 spots, 43.6M bases	RIKEN_CDB, 2010
SRP002028	*Strombus gigas*	Sea snail	cDNA library construction	LS454-4 runs, 286,933 spots, 74M bases	University of Florida, 2010
SRP000456	*Zoarces viviparus*	Viviparous eelpout	Massively parallel pyrosequencing of transcriptome	LS454-3 runs, 378,238 spots, 91.3M bases	Department of Zoolog, 2009

Table 2.5 Some examples of SNP discovery and genotyping in aquaculture and related species.

Species	Common name	Platform/Strategy	Project
Ictalurus punctatus and *I. furcatus*	Channel catfish and blue catfish	EST mining	Linkage map (Kucuktas et al., 2009)
I. punctatus and *I. furcatus*	Channel catfish and blue catfish	EST mining	Inter-specific and intra-specific SNPs identification (Wang et al., 2010a)
I. punctatus	Channel catfish	Affymetrix	Mapping and QTL (ongoing)
Litopenaeus vannamei	Pacific white shrimp	Direct sequencing	QTL study (Zeng et al., 2008)
L. vannamei	Pacific white shrimp	EST mining	SNP discovery (Gorbach et al., 2008)
L. vannamei	Pacific white shrimp	MassArray (Sequenom)	SNP discovery and genotyping, QTL analysis (Ciobanu et al., 2009)
Macrobrachium rosenbergii	Giant freshwater prawn	EST mining and direct sequencing	SNP discovery and genotyping, QTL analysis (Thanh et al., 2010)
Oncorhynchus tshawytscha, *O. nerka*, and *O. keta*	Pacific salmon	EST mining	SNP discovery (Smith et al., 2005)
Oncorhynchus mykiss	Rainbow trout	Reduced representation shotgun sequencing	SNP discovery (Sánchez et al., 2009)
O. mykiss	Rainbow trout	GoldenGate (Illumina)	SNP validation (Sánchez et al., 2009)
Salmo gairdnerdneri	Salmon and steelhead	Dynamic array (Fluidigm)	Populatin differentiation (ongoing)
Salmo salar	Atlantic salmon	EST mining	SNP discovery (Hayes et al., 2007)
S. salar	Atlantic salmon	EST mining/MassArray (Sequenom)	QTL study (Boulding et al., 2008)
S. salar	Atlantic salmon	EST mining	Linkage map and Illumina Infinium BeadChip (Kent et al., 2008)
S. salar	Atlantic salmon	MassArray (Sequenom)	Integration between Linkage map and physical map (Lorenz et al., 2010)
S. salar	Atlantic salmon	iSelect HD (Illumina)	Mapping and QTL (ongoing)

resources. Here, we are not attempting to provide a thorough review; rather, providing a few examples of databases constructed for aquaculture species. Details of the characteristics of these databases, including their web links, are summarized Table 2.6.

Zebrafish

ZFIN (The Zebrafish Information Network) is a web-based zebrafish database established by University of Oregon in 1994 (Sprague et al., 2006; Bradford et al., 2011). It is an essential resource for zebrafish biology comprising of the following primary data types: phenotypes, genotypes, genes, gene expression, functional and phenotypic annotations, anatomical structures, orthology, nucleotide and protein sequence associations, and reagents such as morpholinos and antibodies (Bradford et al., 2011). Its ability to continually update and make data compatible with different types of formats and resources makes it a powerful resource for zebrafish research.

Catfish

The catfish genome database cBARBEL (catfish Breeder And Researcher Bioinformatics Entry Location) is an integrated platform of all ictalurid catfish (*Ictalurus* spp.) molecular data (Lu et al., 2010). At present, the resources available for cBARBEL include: nucleotide sequence (full length cDNAs, ESTs, BAC end sequences), genetic markers, (microsatellites, SNPs) and maps (physical map, linkage map and comparative map). It is expected that the whole genome sequences will soon be integrated upon completion in the near future.

Atlantic Salmon

The Atlantic salmon genome database is perhaps the most comprehensive among aquaculture species. The cGRASP, consortium for Genomic Research on All Salmonids Program, pooled the data from Canadian Genomics Research on Atlantic Salmon Project (GRASP) and the Norwegian Salmon Genome Project together. The salmon data base includes various resources such as linkage maps, physical maps, BAC sequences, BAC end sequences, EST sequences, and repetitive element masking. It is also expected to host the whole genome sequences of the Atlantic salmon upon its completion in the near future.

Fugu

Fugu Genome Project Webpage mainly focuses the update of Fugu genome sequences. The latest edition of Fugu genome assembly is v5 done in January 2010, which was generated by filling some gaps and organizing scaffolds into chromosomes based on the genetic map. v5 assembly comprises 7119 scaffolds covering 393 Mb (Kai et al., 2011). Fugu genetic linkage map and TFCONES (Comparative genomics of transcription factor-encoding genes in human and fugu) can also be found in the website.

Medaka

Medaka Genome Database (M Base) is constructed by the University of Tokyo. The database covers the information of Medaka Expressed Sequence Tag Database (MEBase), Medaka Linkage Map Database (MLBase), and (Medaka BAC Database (MBBase).

Pufferfish

Tetraodon Genome Browser collects *tetraodon* genome sequence analysis results from Genoscope and the Broad Institute of MIT and Harvard. The assembly was performed using the Arachne program.

Stickleback

Stickleback Genome Browser Gateway was created by Genome Bioinformatics Group of

Table 2.6 Some examples of bioinformatic databases for aquaculture and related model fish species.

Species	Website	Information included	Host organization
Zebrafish (*Danio rerio*)	http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg	Mutants, genes, genetic markers, mapping panels, antibodies, etc.	University of Oregon
Atlantic salmon (*Salmo salar*)	http://grasp.mbb.sfu.ca/	Linkage map, physical map, BAC sequences, BES, EST, repetitive element masker	Simon Fraser University
Catfish (*Ictalurus* spp.)	http://www.catfishgenome.org/cbarbel/cgi-bin/search.cgi	Full length cDNA, ESTs, BES, SNP, physical map, linkage map, cMap, alternative splicing	Auburn University
Fugu (*Takifugu rubripes*)	http://www.fugu-sg.org/index.html	Fifth edition of Fugu genome sequence	The Institute of Molecular and Cellular Biology
Marine genomics database	http://www.marinegenomics.org/	ESTs, tools, and other genomic resources of marine species	Clemson University
Medaka (*Oryzias latipes*)	http://earth.lab.nig.ac.jp/~mbase/medaka_top.html	EST, Linkage map, BAC database	University of Tokyo
Pufferfish (*Tetraodon nigroviridis*)	http://www.genoscope.cns.fr/externe/tetranew/	Chromosome browse, Genome assembly V7, Annotation V7.1	Genoscope
Rainbow trout (*Oncorhynchus mykiss*)	http://locus.jouy.inra.fr/cgi-bin/lgbc/mapping/common/intro2.pl?BASE=rainbow	Gene list, polymorphism markers, homology query etc.	INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE
Stickleback (*Gasterosteus aculeatus*)	http://genome.ucsc.edu/cgi-bin/hgGateway?db=gasAcu1	Genome information	UCSC
Tilapia	http://cichlid.umd.edu/CGCindex.html	Linkage maps, physical maps, BAC libraries, cDNA libraries	University of Maryland
Various species	http://www.animalgenome.org/community/other.html	Community and various genome resources	USDA NRSP-8 Project

Table 2.7 Some examples of whole genome sequencing projects with aquaculture and related model fish species.

Species	Genome size (Mb)	Status	Method	Depth	Release date	Center/consortium
Atlantic cod (*Gadus morhua*) NEAC_001	930	In progress	WGS	30X		Genofisk
Beira killifish (*Nothobranchius kuhntae*)	5.2	Assembly	Clone-based	0.005X	03/17/2009	Fritz Lipmann Institute, Germany, Jena
Blue mbuna (*Labeotropheus fuelleborni*) Domwe Island	69.3117	Assembly	WGS	0.15X	06/20/2008	Cichlid Genome Consortium
Brichardi (*Neolamprologus brichardi*) Inbred	1000	In progress	WGS	200X		Broad Institute
Burton's haplochromis (*Haplochromis burtoni*)	1000	In progress	WGS	200X		Broad Institute
Channel catfish (*Ictalurus punctatus*)		In progress	WGS	70X		Auburn University & USDA
Chinese longsnout catfish (*Leiocassis longirostris*) LELOWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Copadichromis eucinostomus (*Mchenga conophoros*)	71.3915	Assembly	WGS	0.15X	06/20/2008	Cichlid Genome Consortium
Elephant shark (*Callorhinchus milii*)	0.647131	Assembly	WGS	1.4X	01/05/2007	Institute of Molecular and Cell Biology, Singapore
European seabass (*Dicentrarchus labrax*) Adriatic clade, male 57	98.2281	Assembly	WGS & clone-based	2X	02/12/2010	European seabass sequencing consortium
Flag Rockfish (*Sebastes rubrivinctus*)	1000	In progress	WGS	44X		University of Southern California
Floridalancelet (*Branchiostoma floridae*)	831.201	Assembly	WGS	11.5X	04/24/2008	DOE Joint Genome Institute
Freshwater snail (*Biomphalaria glabrata*)		In progress				Genome Sequencing Center (GSC) at Washington University (WashU) School of Medicine
Fugu (*Takifugu rubripes*)	332.348	Assembly	WGS	8.7X	08/22/2002	Fugu Genome Sequencing Consortium

Golden Mbuna (*Melanochromis auratus*) Domwe Island	66.5029	Assembly	WGS	0.12X	06/20/2008	Cichlid Genome Consortium
Grass carp (*Ctenopharyngodon idella*)		In progress				Chinese Academy of Sciences
Grass carp (*C. idella*)		In Progress				School of Biological Sciences, University of Hong Kong, Pokfulam Road, Hong Kong, SAR, China
Half smooth tongue sole (*Cynoglossus semilaevis* Gunther)		In progress	WGS			Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Science
Hong Kong catfish (*Clarias fuscus*) CLFUWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Indonesian coelacanth (*Latimeria menadoensis*)	2400	In progress	WGS & clone-based			Broad Institute
Japanese killifish (*Oryzias latipes*) Hd-rR	800	In progress	WGS	5X		Keio University, Japan
Japanese killifish (*O. latipes*) Hd-rR	700.37	Assembly	WGS		04/19/2006	Medaka genome sequencing project
Japanese killifish (*O. latipes*) HNI	585.155	Assembly	WGS		05/07/2007	Medaka genome sequencing project
Japanese sea bass (*Lateolabrax japonicus*) LAJAWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Lake Victoria cichlid (*Pundamilia nyererei*)	1000	In progress	WGS	200X		Broad Institute
Lake Victoria haplochromine (*Paralabidochromis chilotes*) Lake Victoria		In progress	Clone-based			Broad Institute
Little skate (*Leucoraja erinacea*)		In progress				Genome Sequencing Center (GSC) at Washington University (WashU) School of Medicine
Little skate (*L. erinacea*)	3420	In progress	WGS	26X		North East Cyberinfrastructure Consortium

(*Continued*)

Table 2.7 (*Continued*)

Species	Genome size (Mb)	Status	Method	Depth	Release date	Center/consortium
Nile Tilapia (*Oreochromis niloticus*)	3000	Assembly	WGS	235X	01/28/2011	Broad Institute
Ocean coelacanth (*Latimeria chalumnae*) Wild caught	3000	In progress	WGS	150X		Broad Institute
Pufferfish (*Tetraodon nigroviridis*)		In progress				Broad Institute
Purple sea urchin (*Strongylocentrotus purpuratus*)	907.094	Assembly	WGS & clone-based	8X	04/07/2005	Baylor College of Medicine
Red Sea Urchins (*Strongylocentrotus franciscanus*)		In progress		1X		Baylor College of Medicine
Sea lamprey (*Petromyzon marinus*)		In progress				Michigan State University
Sea lamprey (*P. marinus*)		In progress			09/30/2010	Genome Sequencing Center (GSC) at Washington University (WashU) School of Medicine
Sea walnut (*Mnemiopsis leidyi*) wild caught	150	In progress	WGS	10X		National Human Genome Research Institute (NHGRI)
Siberian prawn (*Exopalaemon modestus*) EXMOWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Slate pencil urchin (*Eucidaris tribuloides*)		In progress				Baylor College of Medicine
Stickleback (*Gasterosteus aculeatus*)	446.611	Assembly	WGS		02/01/2006	The Genome Assembly Team
T. nigroviridis	342.403	Assembly	WGS	6X	05/14/2004	Genoscope

Tigerfish (*Rhamphochromis esox*) Otter Point	69.8333	Assembly	WGS	0.16X	06/23/2008	Cichlid Genome Consortium
Turbot (*Scophthalmus maximus*) SCMAWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Turquoise killifish (*Nothobranchius furzeri*) GRZ	5.3	Assembly	WGS	0.005X	03/17/2009	Leibniz Institute for Age Research - Fritz Lipmann Institute (FLI)
Turquoise killifish (*N. furzeri*) MZM-0403	5.3	Assembly	WGS	0.005X	03/17/2009	Fritz Lipmann Institute, Germany, Jena
White Amur bream (*Parabramis pekinensis*) PAPEWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Whiteleg shrimp (*Litopenaeus vannamei*) Hainan breeding family	2445	In progress				Chinese Academy of Sciences
Yellowhead catfish (*Pelteobagrus fulvidraco*) PEFUWH01	17	In progress				Institute of Bioinformatics, Anhui Normal University, China
Zebrafish (*Danio rerio*) Tuebingen	3112.62	Assembly	WGS & clone-based	7X	01/28/2005	Wellcome Trust Sanger Institute
Zebrafish (*D. rerio*) Wild		In progress	Clone-based			University of Manitoba
Zebra Mbuna (*Maylandia zebra*)	1000	In progress	WGS	200X		Broad Institute
Zebra Mbuna (*M. zebra*) Mazinzi Reef	76.9832	Assembly	WGS	0.17X	06/21/2008	Cichlid Genome Consortium

UC Santa Cruz. Specified genome information can be traced by the accession number.

Rainbow Trout

Rainmap Database (edition 2.0) shows the rainbow trout genes, gene loci, markers, polymorphisms, homology, and sequence information.

Whole Genome Sequence Assemblies

With the rapid development of sequencing technologies, the sequencing capacity has been dramatically increased, while the sequencing cost has been drastically reduced. Accordingly, whole genome-based selection for aquaculture species will soon be technically feasible. In Table 2.7, we have summarized the sequencing status for around 50 species from NCBI genome database. Apparently, whole genome sequences will soon become available for many species that will be the most powerful genome resource for functional genomics research.

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Chapter 3

Production, Growth, and Insulin-Like Growth Factor-I (IGF-I) Gene Expression as an Instantaneous Growth Indicator in Nile Tilapia *Oreochromis Niloticus*

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Abstract: Worldwide, rapid expansion of the market for *Oreochromis* spp. (tilapia) has increased the incentive for culturists to optimize the profitability of production techniques for these fishes. The establishment of best management practices for tilapia production has been slow, in part because they are omnivorous and relatively easy to grow. Their wide distribution in subtropical and tropical areas and the ease of adaptation to different culture methods have contributed to the highly variable approaches that are used to cultivate tilapia commercially. Ongoing refinement of the efficiency of tilapia culture in response to environmental, nutritional, and genetic variables is reliant on accurate assessment of growth rates. We describe herein a molecular method for the rapid assessment of the growth status of these fish. Earlier trials of culture conditions have been dependent on expensive commercial-scale production trials and labor-intensive physical measurements of growth, but expression of the insulin-like growth factor-I (IGF-I) gene provides a nearly instantaneous indicator of the growth status of these fishes. The relative accuracy and efficiency of quantifying the hepatic mRNA (messenger RNA) for this growth regulatory compound and its applicability as a growth indicator or marker in tilapia are discussed. We conclude that IGF-I mRNA abundance is suitable as an alternative approach to the assessment of growth during trials of the relative effectiveness of experimental culture conditions.

Introduction

Insulin-like growth factor-I (IGF-I) is a critically important mediator of growth in vertebrates. Studies in IGF-I knockout mice show that IGF-I is responsible for much of postnatal growth (Lupu et al., 2001), and the presence or absence of a single IGF-I nucleotide polymorphism is a major determinant of body size in domestic dogs (Sutter et al., 2007). The presence of circulating IGF-I is associated with anabolic states, and hormone abundance has been used as an indicator of nutritional status in humans (Swenne et al., 2007). As a result

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

of the close association of IGF-I with nutritional status and the growth that is contingent on it, IGF-I quantification has been applied in the diagnosis and investigation of human eating disorders (Schneider et al., 2006). The speed of detectable changes in IGF-I production and the rapidity of responses to changes in nutritional status are among the reasons why this hormone has been used as a "marker" of malnutrition in humans, and applications among other vertebrates in agriculture have recently begun to emerge. Serum IGF-I concentrations display positive correlations with body weight and are moderately to highly heritable in cattle (Davis and Simmen, 2006). In this chapter, we provide a synopsis of the value of tilapia to aquaculture, and introduce the value of IGF-I as an indicator of growth status in cultured fish species.

Aquaculture and Health Benefits

The regulation of growth in fish is a subject of intense interest, since supply and demand factors have contributed favorably to the growing incentive to cultivate fish. Demand for seafood is increasing, with global seafood consumption on the rise both because of increased consumption per capita (FAO, 2009) and increasing world population.

The consumption of fish per capita has been relatively stable, but is steadily increasing. This increased consumption of fish on the per individual basis is partly due to the real and perceived health advantages attributed to edible fishes and invertebrates compared with other commonly available sources of protein. The evidence favoring health benefits of dietary fish intake is overwhelming. Examples include a 16-year study demonstrating reduced risk of coronary heart disease in human subjects in response to increased fish ingestion (Hu and Willet, 2002). Many of the benefits of fish consumption have been attributed to the relatively low amounts of saturated fats and the high proportion of omega-3 fatty acids in fish and fish oils. Dietary omega-3 fatty acids reportedly reduce the rates of all-cause mortality, cardiac and sudden death, and possibly stroke (Wang et al., 2006).

Positive evidence of the health benefits of eating fish contrasts with some inherent risks of seafood. For example, the consumption of fish has been associated with increased likelihood of exposure to bioaccumulated environmental contaminants, such as mercury and PCBs. Both the US Environmental Protection Agency and the Food and Drug Administration have consequently recommended restricted rates of intake of fish (see Kris-Etherton et al., 2002), but opinions with regard to the overall balance of benefits and hazards of dietary fish vary. A quantitative evaluation of the relative risks from contaminants of fish consumption led to the conclusion that the nutritional benefits outweigh risks for both normal and pregnant subjects (Mozaffarian and Rimm, 2006). Those authors have concluded that the dietary value of fish is significant enough that both aquaculture and capture fisheries should be continued, with refinements aiming toward sustainability. Although the bioaccumulation of undesirable materials in the marine food chain is a known hazard of fisheries, this is viewed as a completely avoidable problem for aquaculturists. Reductions, or the elimination, of wild materials in feeds used in mariculture can circumvent the problem of bioaccumulated contaminants.

The rising demand for cultured fishes has contributed to a growing interest in cultivated seafood. The annual per capita consumption of fish has been increasing steadily (The World Bank, 2006) despite the leveling off of supplies from capture fisheries. Concerns have been raised about the cultivation of genetically engineered and hormone-treated fishes, as well as the availability of farmed aquatic animals that contain antibiotics and dyes (Center for Food Safety, 2005). A general preference for wild fishes appears to be based on nutritional advantages linked to the wild diet, but the prospects of maintaining wild animal populations capable of supporting more than 6 billion humans do not look promising. In addition to the conservation

and safety issues mentioned in the preceding text, the predictability of capture fisheries and their dependence on fossil fuels add to concerns about profitability and sustainability. The Food and Agriculture Organization (FAO) estimated in 2004 that half of the world's fish populations were already fully exploited and an additional one-fourth were already overexploited (FAO, 2004). The relative proportions of global fisheries resources that are considered to be underexploited are on a declining trend, while the proportions that are either fully exploited or overexploited continue to increase (FAO, 2009). For these reasons, and despite a growing demand for seafood products, the conclusion was reached in 2004 that "the maximum wild capture fisheries potential from the world's oceans has probably been reached" (FAO, 2004, 2009).

Because of the reduced wild supply and consumer demand for seafood, global aquaculture has surged, with production increasing at an average annual rate of 10% (The World Bank, 2006). The United States plays a relatively small role in global aquatic farming; developing countries account for 90% of world aquaculture production (FAO, 2009). This has resulted in a huge and increasing trade deficit for seafood in the United States. The majority of seafoods consumed in the United States are cultured or captured in other countries. The statistics of the United Nations Food and Agriculture Organization (FAO, 2009) indicate that over $13 billion worth of seafood were imported into the United States in 2006 alone. Clearly, aquaculture has a great potential in providing the healthy protein sources and meeting the demand for seafood consumption.

Economic Prominence of Tilapia

A large and steadily increasing portion of the global aquaculture market share is held by tilapia (El Sayed, 2006), largely because of its versatility and great adaptability as culture subjects. Tilapia are omnivorous fishes that adapt very well to a wide range of production strategies, growing rapidly in ponds, cages, tanks, and polyculture systems. Because they are opportunistic feeders, they also adapt well to varied diets and feeding schedules. The diets for tilapia are also substantially cheaper because of the lower requirements of tilapia for animal protein, particularly supplied in the form of fishmeal. Fishmeal is among the most costly components of milled feeds used in the production of aquacultured species, especially so for carnivorous fishes. The social nature of tilapia and their higher resistance to disease also contribute to their suitability for cultivation at relatively high densities.

The Nile tilapia is a favored subject for mass culture because of its superior growth rates on virtually any feed. Other members of this family also offer considerable potential for domestication; consequently, an improved understanding of growth and of the most favorable conditions for the culture of tilapia of the genus *Oreochromis* has broad implications for human nutrition and welfare.

The emergence of tilapia as a highly valued farm animal is accompanied by increasing market acceptance among consumers. Tilapia were once favored primarily in developing countries, and viewed in the industrialized world as a low-cost dietary alternative to a better grade of fish, but they continue to gain popularity. Tilapia have displaced a substantial market share among more expensive fishes, including species with established popularity in upscale, marine seafood markets. As supplies of wild fishes have slumped and prices have increased, the availability and quality of cultured tilapia have been improving. Importantly, the consistently good quality of marketed tilapia is contributing to the elimination of earlier stigmas of it being a poor quality or "junk" fish (Engle, 2006). It has been reported on occasion that tilapia flesh has been marketed illegally as the meat of other, pricier species—even including the sale of tilapia as sashimi-grade *white tuna* (Schwartz, 2008). The revelation that tilapia

meat can pass for a premium grade of tuna for sashimi—a scam that was confirmed only by the analysis of DNA sequences—is a convincing but somewhat bizarre testimony to the quality and gustatory appeal of tilapia products. Tilapia meat is now imported legitimately and accepted in Japan for consumption in the form of sushi and sashimi (Fitzsimmons and Posadas, 1997).

A single study recently touched off a debate about the nutritional value of tilapia. The conclusion that the ratio of omega-3 to omega-6 fatty acids was unfavorably skewed toward the latter in tilapia (Weaver et al., 2008) led to quotes from that article in the media that "the inflammatory potential of hamburger and pork bacon is lower than . . . tilapia." (Welland, 2008). This was considered by some to be more an example of inflammatory journalism than of scientific facts. Some have argued that fatty acid ratios are not particularly good determinants of overall food quality. Others have argued that fatty acid ratios are products of the farmer's selection of feeds or culture temperatures and, therefore, are highly subject to variation in an omnivorous species. The net effect of the extensive media backlash to the bacon and donut comparison appears by consensus to have been a general reaffirmation of the positive balance of nutritional qualities in tilapia (e.g., see Welland, 2008). Overall, the omega-3 fatty acid content of tilapia is far less than that of salmon, but so too is the total amount of fat and the amount of saturated fat. It is probably safe to say that most nutritionists view tilapia as a worthwhile component of a healthy, balanced diet.

During the emergence of tilapia as a large-scale aquaculture product, parallels are frequently drawn between tilapia and poultry production. Poultry production is an exemplary model for up-and-coming species like tilapia, and much of what has been learned and accomplished with poultry has been at least partially dependent on exhaustive nutritional studies coupled with growth trials (e.g., see Summers and Fisher, 1961). The high efficiency and low cost of domestic poultry production reflect superior feed conversion, nutrition, genetic improvements, and refinements of production techniques. Similarly, improvements in tilapia husbandry methods and the elaboration of superior genetic stocks (Ng and Hanim, 2007) have contributed to declining production costs. The consequent upgrades in product quality and availability have contributed to steady gains in market share. It is not unusual to hear tilapia referred to as the "Aquatic Chicken" (see Coward and Little, 2001).

The continually expanding scale of the tilapia production industry has led to several "coming of age" events, signaling the stepwise emergence of a new domesticated species. A US company with extensive aquaculture activities in China—HQ Sustainable Maritime Industries—has produced a *Tilapia Bill of Rights* that promotes healthy and humane cultivation practices, with a clear goal of sustainability (Reuters, 2008). That bill lists acceptable production parameters including the elimination of fishmeal-based feeds and prohibition of growout in cages; it also includes a proclamation that hormones will not be used for the generation of monosex crops.

The emergence and refinement of aquaculture technology are contingent at least in part on the optimization of cost-effective methods to promote accelerated growth, and the measurement of growth instantaneously would streamline progress in that area. It is for these reasons that we propose in this chapter the quantification of IGF-I gene expression as a short-term, nearly instantaneous indicator of the growth status in tilapia.

Growth Trials

The refinement of tilapia culture methods rests squarely on our knowledge of the biology and nutrition of these species, but there is at present no agreement on a single set of "Best Management Practices." That may be partly a reflection of the wide distribution of tilapia culture

operations, and the flexibility of their physical and nutritional requirements. The suitability of a variety of different culture methods is a result of the inherent adaptability of these omnivorous animals, as well as their wide distribution.

Rising costs of energy and fishmeal have contributed some urgency to the need to refine tilapia culture methods. Testing and experimentation continue to focus on the elaboration of efficient, large-scale production methods in order to help meet human nutritional needs at a modest cost. The implementation of new farming methods cannot be extrapolated from laboratory results without considerable guesswork, since relationships seen under controlled conditions in an aquarium room often do not apply directly on the scale and under the environmental conditions present on farms. We are learning, for example, that the social conditions under which tilapia are reared have profound influences on the dynamics of their growth processes (Vera Cruz and Brown, 2007). Tests in aquaria typically have a scaled-down social structure as opposed to farm production. Farm trials are typically carried out to test for the most acceptable and cost-effective production methods on a commercial scale. Trials of this sort address costs as determined by the consumption of feeds, fuel and other resources used, labor and energy costs, space and equipment required, and product volume and quality or, a "partial economic analysis." A typical tilapia growout cycle takes 4 months in the Philippines, where the harvest of relatively smaller fish is preferred (Eknath et al., 1998). Growout trials elsewhere in intensive recirculating systems, ponds, or cages typically last 4–9 months or more (e.g., Little et al., 2003). Because statistically valid interpretation of growth data depends on the comparative performance of replicated treatment and control groups, the magnitude and cost of even a simple farm-scale growout trial can be high. With new diets under development and methods being adapted for environmentally sound production of various strains of fish in different regions, the need for accurate quantification of growth performance in the testing of experimental aquaculture methods is substantial. Therefore, a rapid, reliable, and cost-effective assessment of the tilapia growth rate appears to be a tool with a high degree of potential utility; it could help reduce our dependence on costly and time-consuming farm-scale "weigh and measure" type growout trials. Molecular bioindicators can be particularly of interest because they not only provide indication to growth but may also suggest molecular mechanisms underlining variations in growth rates.

We have described a rapid and sensitive means of determining the growth rate that could have applications not only in the optimization of diets and culture parameters but also in the genetic refinement of domestic tilapia stocks (Vera Cruz et al., 2006). Selection processes for the evaluation of genetically improved strains of tilapia are also based, in part, on the results of growth trials, in which performance is typically measured over months (Hussain et al., 2000; Ng and Hanim, 2007). Therefore, it appears likely that genetic selection processes could also be streamlined through the use of an accurate molecular indicator of growth rates.

The Relationship of Insulin-Like Growth Factor-I (IGF-I) to Growth

IGF-I is the primary endocrine or paracrine mediator of growth in vertebrates. This hormone is at the tail end of the cascade of processes that transduce environmental, nutritional, and social information into somatic growth responses in tilapia, as in other vertebrate animals (Duan, 1997, 1998; Shepherd et al., 2006; Picha et al., 2008b). Favorable temperatures, adequate nutrition, stimulatory photoperiods, etc. promote hypothalamic and pituitary reactions including the release of growth hormone. Circulating growth hormone, or somatotropin, causes the systemic release of IGF-I (and possibly IGF-II) as well as its production locally in many tissues. The potential value

of the measurement of IGF-I in growth assessment is underscored by the fact that this hormone directly stimulates anabolic responses in peripheral tissues, mediating most of the growth-promoting actions of growth hormone (Shepherd et al., 2006).

One approach to the use of IGF-I as an indicator of growth status is to determine its gene expression levels in tissues. We have examined this by measuring the amount of mRNA encoding for IGF-I in a small hepatic tissue sample using TaqMan® real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and a highly specific molecular probe (see Vera Cruz et al., 2006). IGF-I is a polypeptide that is not stored in granules, but rather is secreted as it is produced. Hence, its gene expression or mRNA levels are likely to reflect the amount of protein produced by the tissue. Since the liver is thought to be the primary source of circulating IGF-I, the quantification of hepatic IGF-I mRNA levels is a good candidate for assessing the overall activity of this important growth factor, without having to rely on measures of circulating hormone. Currently, radioimmunoassay (RIA) is the best available method for measuring circulating IGF levels (e.g., see Reineke et al., 1992; Picha et al., 2008a). This method requires the use of radioisotopically labeled hormone along with an IGF-I specific antibody. While the antibody derived from fish or humans may work for a variety of species, the use of radioisotopically labeled hormone involves some hazards as well as substantial regulatory issues. Indeed, many regions of the world do not allow use of radioisotopes, so the applicability of the radioimmunoassay procedure is quite limited globally.

Tissues other than liver also produce IGF-I, and this paracrine source of hormone may also be important in eliciting tissue and organismic growth. The measurement of IGF-I mRNA by qRT-PCR is the most sensitive technique for the quantification of low-abundance mRNA obtained from limited tissue samples. The assay method is dependent on the use of a tilapia-specific primer for the nucleotide encoding IGF-I, and quantification of triplicate samples of the mRNA for IGF-I by spectrophotometry, compared with values derived from a standard curve of known concentrations, or to the expression of a housekeeping gene that is assumed to be constitutively expressed and not regulated by the test conditions. The IGF-I mRNA is quantified by substituting the generated threshold cycle (C_t) value in the equation derived from the standard curve (plot of the log of initial target copy number vs. C_t). The concept of the C_t uses fluorescence-based RT-PCR. Fluorescence values are recorded by the instrument during every cycle of the amplification reaction. This value is representative of the amount of IGF-I mRNA amplified. The more templates present at the beginning of the reactions, the fewer cycles are needed to reach the C_t value, which is the first cycle with detectable fluorescence in relation to an internal standard.

This approach has some advantages and disadvantages. This molecular method requires training and access to sophisticated equipment that may not be readily available in some places. Although the technique can be used for measuring IGF-I gene expression in any tissue, we have limited its application to measurements in the liver since it is the presumed source of the circulating hormone. Nonetheless, tissues other than liver also produce IGF-I, and this paracrine source of hormone may also be important in eliciting tissue and organismic growth (Picha et al., 2008a).

A distinct advantage of the measurement of IGF-I gene expression is that it reflects change in the growth status of an animal very quickly. The IGF-I gene expression method gives a summary value for growth status that is representative of and highly correlated with the growth rate (Vera Cruz et al., 2006), and provides a clear means of sorting out the interactive mix of environmental, nutritional, and social factors that influence growth. Initial results with the detection of changes in IGF-I gene expression have revealed strong responses to increased temperatures and dietary regimens. The expression of the hepatic IGF-I gene was

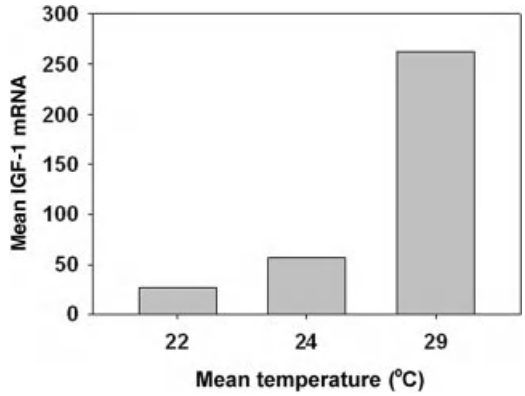

Figure 3.1 Relative IGF-I mRNA as quantified in *Oreochromis niloticus* hepatic tissue samples from fishes held at three different temperatures, compared with the level found in fasted fish. (Redrawn from Vera Cruz et al. (2006) with the permission of the authors.)

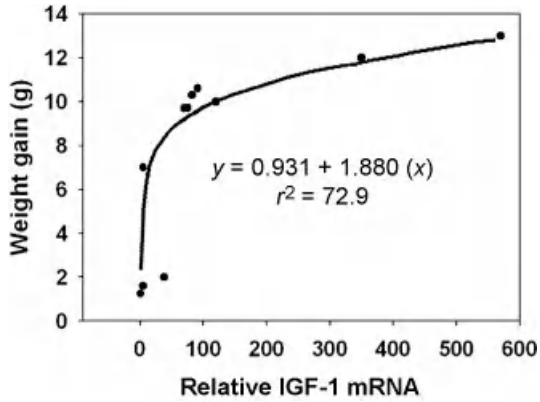

Figure 3.2 Correlative relationship ($R^2 = 72.9$; $p < 0.05$) of weight gain resulting from various feeding regimens with relative concentrations of IGF-I mRNA detected in *Oreochromis niloticus* hepatic tissue samples. The positive correlation between weight gain and IGF-I mRNA suggests the possible value of this molecular method as an indicator of growth. (Redrawn from Vera Cruz et al. (2006) with the permission of the authors.)

sharply elevated in fish fed the same rations but held at elevated temperatures (Figure 3.1). These tilapia also showed enhanced feed conversion and growth rates in response to alterations in temperature (Vera Cruz et al., 2006). A positive correlation was also found between diet as an independent variable and resultant changes in measurable hepatic IGF-I mRNA (Figure 3.2; see Vera Cruz et al., 2006). A weaker association was seen between IGF-I mRNA and the growth rate as altered by experimental photoperiods (Vera Cruz and Brown, 2009). In each of these relationships, moderate variance was seen—IGF-I gene expression did not have a linear correlation with any of the environmental or dietary parameters. The relationship of growth to IGF-I gene expression appears to be a classic, sigmoid curve (Figure 3.2). One important source of variance in these data was the social status of the fish; even under identical experimental conditions within the same treatment groups, subordinate male fish had consistently lower growth rates and IGF-I gene expression values compared with dominant male fish (Vera Cruz and Brown, 1992).

Although the social status and hierarchical rank of fish are rarely considered on a commercial production scale, it appears likely that dominance could significantly affect growth and production rates. Competition for food has been associated with agonistic behavior during feeding in *Sparus aurata*, with hierarchical dominance rank profoundly affecting the growth rate (Goldan et al., 2003). Competition for food appears to be applicable to at least some degree in larger schools of *S. aurata* and other cultured species (Goldan et al., 2003). Dominance hierarchies also figure strongly in the establishment of patterns of dominance and competitive aggression in the culture of large numbers of a range of larval fish species (Kestemont et al., 2003).

Practical Utility of IGF-I mRNA as an Indicator for Growth Rate

Improvements over the conventional method of determining the growth rate by measuring body mass after a period of time could involve

quantification of any of a number of potential indicators: somatotropin releasing factors, growth hormone, growth factors, and the various receptors that bind these hormones and the respective RNAs that encode for each of them (reviewed by Picha et al., 2008a). Other indices have been used, such as the ratios of RNA to DNA concentrations in tissues, since the proportions of these nucleotides change in tissues that undergo increased gene expression associated with the anabolic state (Mercaldo-Allen et al., 2008). Total RNA and DNA are discernable and their ratio is commonly used in the rapid assessment of the growth rate in fishes (Chicharo and Chicharo, 2008) and particularly in larval fishes (Sharma et al., 2006). The precision and reliability of RNA/DNA ratios as an indicator can be compromised during periods of intermittent growth (Westerman and Holt, 1994), and can be subject to temperature-dependent changes.

Quantification of the nucleotide encoding for IGF-I is relatively rapid and precise in comparison with the aforementioned methods. The mRNA for IGF-I is detected through the use of a specific molecular probe, which is based on the known sequence of the IGF molecule in the Nile tilapia (Vera Cruz et al., 2006). A qRT-PCR assay is used to quantify the mRNA for IGF-I in Nile tilapia (Vera Cruz et al., 2006). The accuracy of this method as a growth indicator is formidable; correlations of the abundance of IGF-I mRNA with the growth rate (Figure 3.2) appear to be stronger and more consistent than are correlations of growth with circulating growth hormone levels (Duan, 1997, 1998; Picha et al., 2008a). The assay for IGF-I mRNA also offers some practical advantages over conventional assessments of growth; it can be done in a single day at a cost that is a small fraction of the net expense of large-scale growth trials ending with weighing and measuring activities.

Conclusions

Growth rate is by far the most important trait in aquaculture. The long-term sustainability of aquaculture relies on genetic enhancement of growth rates, particularly through the application of functional genomic approaches or whole genome-assisted selection. Before such genetically enhanced lines become available, indicators for fast growth can help aquaculturists to achieve their goals of making proper decisions in selection of brood stocks. IGF-I, a major factor regulating growth in tilapia as well as in other animal species, provides a useful indicator for growth in tilapia. Although the initial cost of equipment and the training required to measure IGF-I mRNA is substantial, the speed, efficiency, precision, and accuracy of this mode of testing as a growth rate indicator suggest a high degree of practical utility. In the case of the Nile tilapia, the IGF-I gene has been cloned and its nucleotide sequence is published (Vera Cruz et al., 2006). Both the continued testing of culture methods and the management of genetically select stocks are likely to benefit from the availability of this rapid means of assessing growth status.

The rapid response of IGF-I genes to growth-promoting conditions is a significant advantage of this approach. Consistently, our studies have shown that significant increases in gene expression occur within 2 days of exposure to stimulatory environmental or nutritional conditions. That provides a huge temporal and cost advantage to researchers evaluating experimental culture conditions, since positive results are apparent long before they would be using biomass or length as an indicator. The conventional approach has been to grow a large number of fish under one or more sets of controlled conditions, and after several months of treatments, to weigh and/or measure them. We have also compared growth, uniformity, and market value of tilapia produced after several months under alternative production methods (Bolivar et al., 2006). The time involved and cost of these experiments are formidable; it has been our experience that the examination of a single variable could often take up an entire year. In contrast, the quantification of IGF-I gene activity can be carried out in a few animals immediately after exposure

to a treatment, and data can be obtained in several days. Therefore, the use of this molecular technique should accelerate the speed of decision-making for fish culturists. It certainly will allow the screening of optimal growth parameters prior to conducting longer term production trials, which are costly and time and resource intensive.

The improvement of methods and strains of fish used for conventional fish farming are not the only processes that are likely to benefit from a rapid and precise means of assaying growth status. Aquaculture interests could shift to the use of low- to moderate-priced fishes for the cultivation of specialized fishes with customized nutritional qualities; for example, relative balances of fatty acids for dietary reasons. Growth trials will undoubtedly be needed to test and confirm the effectiveness of new diets for such specialized strains of fishes. Both nutritional and aquaculture sciences will be required to generate products with desired nutritional profiles, and the speed and efficiency of growth in response to variable culture parameters are likely to remain highly ranked among the practical interests of producers.

Acknowledgments

This work was generated from joint efforts of the National Oceanographic and Atmospheric Association (NOAA), North Carolina State University, Central Luzon State University, and the Aquaculture and Fisheries Collaborative Research Support Program (AFCRSP), and was funded in part by USAID grant number EPP-A-00-06-00012-00. The Aquaculture and Fisheries CRSP accession number is 1361. The opinions expressed herein are those of the authors and do not necessarily reflect the view of these agencies.

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Chapter 4

Gene Expression Pattern during European Sea Bass Larvae Development: Impact of Dietary Vitamins

David Mazurais, Maria Darias, Ignacio Fernández, Chantal Cahu, Enric Gisbert, and Jose-Luis Zambonino-Infante

Abstract: Substantial progress in pisciculture has been made in the last two decades, but many unidentified parameters that can still affect juvenile quality and, therefore, limit the cost-effectiveness of the production represent an industrial bottleneck. The most critical phase of the whole production cycle is probably the larval period. To control this phase of the production cycle, it is essential to decipher the ontogenetic mechanisms and underlying regulations governing the harmonious development of the larvae in order to understand the disruptions induced by environmental/nutritional factors. It is now well admitted that the regulation of the developmental processes involves the control of the expression of genes whose products influence the biological functions related to ontogenetic pathways. The recent development of the genomic resources in European sea bass allows the investigation of a large panel of genes involved in different biological processes and notably to study simultaneously the expression of several thousands of genes. This chapter reviews the recent data related to larval development obtained through transcriptomic approach in sea bass and provides an overview of the effects of dietary vitamins on the expression of several key genes acting on the skeletogenesis processes.

Introduction

At hatching, most of the organs of marine fish larvae are not fully developed. Marine fish larvae hatch much earlier in their development than other vertebrates, suggesting that the spatiotemporal sequences of their organogenesis and morphogenesis are quite different from those of higher vertebrates. Those processes, initiated during embryogenesis, have, thus, to proceed during larval development in order to ensure the ontogenesis of organs and associated physiological functions essential for the survival of individuals in the natural habitat. This phase of larval development leading to the juvenile stage is certainly the most critical in the life cycle of fish. The larva is vulnerable not only to predators in the natural environment but also to environmental factors that can impinge on its ontogenesis and, therefore, can impact their survival. In aquaculture, in spite of the many progress made to control environmental cues, this phase of larval development can still suffer from substantial mortality or skeletal malformations, impairing the overall performance of the rearing production.

The larval period is still poorly understood even though increasing amount of work have revealed the key roles played by nutrients

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

and, especially vitamins, during fish larval development (for review, see Oliva-Teles, 2000; Shields, 2001; Takeuchi, 2001; Hamre et al., 2010). However, as mentioned by Power et al. (2008), the main difficulty concerning the identification of the causes and the mechanisms involved in these abnormalities is the lack of information on the sequence of developmental and physiological changes that underlie the normal larval development in fish, as well as the crosstalk among the developmental changes. The recent development of genomic resources, including ESTs (expressed sequence tags) and microarrays, for European sea bass (*Dicentrarchus labrax*) allows the simultaneous investigation of the expression of several thousands of genes and consequently a global and integrated view of molecular, cellular, and physiological processes involved in the larval development of a marine fish species. This chapter reviews the recent data related to European sea bass larval development obtained through transcriptomic approach and gives an overview of the effects of dietary vitamins on the expression of several key genes.

Larval to Juvenile Development: Morphological Aspects

The development of fish from a fertilized egg to a larva and then to a juvenile is species specific. Hatching can thus occur at different embryonic stages and direct or indirect developments are considered to be the two ways to reach the juvenile phase (Youson, 1988; Balon, 1999). Direct ontogeny concerns very few species, such as sand-dwelling clades (*D. compressiceps* and *Tramitichromis intermedius*), with post-embryonic fish resembling the juvenile or adult form even if they are smaller in size and sexually immature (Kasyanov et al., 1998). On the contrary, indirect development is characterized by an intermediate larval period during which a small post-hatched organism, with rudimentary organs, will metamorphose into a juvenile with an almost definitive phenotype. The duration of such larvae developmental period as well as the extent of morphological remodeling depends also on fish species. Developmental staging schemes have been defined in different species based on external phenotypes. The most dramatic morphological changes are observed in flatfish species (Division: Teleostei; Order: Pleuronectiformes), which undergo metamorphosis from a symmetrical pelagic larva to an asymmetric benthic juvenile. In pelagic fishes, although exterior phenotype changes are less evident, organisms exhibit several morphological modifications. In European sea bass, which normally hatch at three to four days post-fertilization when reared at 15–17°C (Saka et al., 2001) the eyes start to pigment, the mouth opens, and the pectoral fins start to be formed within the five days post hatching (dph; Barnabé et al., 1976). The transition from endogenous to exogenous feeding period is thought to be completed when larvae have no more visible yolksac reserves (15 dph). From 20 dph, the vertebrae are visible and the pigmentation continues to develop. The primary fin becomes blurred at 32 dph and the caudal, anal, and dorsal fins are formed. Around 35 dph appear the pelvic fins and then the larva shows the morphological characteristics of the adults (summarized in Figure 4.1). Morphogenetic events occurring during larval development are correlated with new swimming performance, which is indeed necessary

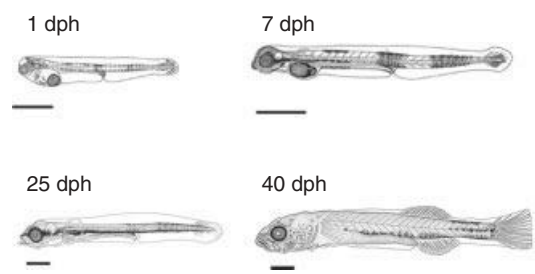

Figure 4.1 Schematic drawing showing different larval stages of European sea bass reared at 20°C. "dph" means days post-hatching. The scale bar corresponds to 1 mm.

for fish larvae to catch preys for feeding and to escape from predator as well. The successful passage through early larval stages is also associated with the maturation of key physiological functions as well as metabolic systems (Falk-Petersen, 2005).

Despite the fact that the molecular and cellular mechanisms buffer organisms from environmental cues, disturbance of the ontogenical process by excessive stressors could negatively impact on morphogenesis events and the chances of the larvae to survive (Hamdoun and Epel, 2007). In the wild, malformed animals rarely survive because of the forces of natural selection, whilst in hatcheries, larvae with many morphological anomalies survive mostly because of the absence of predators and an excess of feed resources. Even if the nature of specific deformities could be species specific, particularly for flatfish, whose metamorphosis includes eye migration and pigmentation changes, most of the related morphological disruption in larvae result from perturbation in the skeletal developmental process (Zambonino-Infante et al., 2005). The most common skeletal deformities observed in hatcheries are spinal malformations (lordosis, scoliosis, kyphosis, fusion and compression of vertebrae, and coiled vertebral column), deformed operculum, and head (jaws and neurocranium) malformations. Several identified parameters could influence this development, including, for example, inappropriate hydrodynamics in the tank, water temperature, light intensity, or salinity (Johnson and Katavic, 1984). Inflammation associated with diseases originated from bacteria or virus can lead to deformities as well (Madsen and Dalsgaard, 1999; Gil-Martens, 2010). Nutritional factors, which will be discussed later on in this chapter, have also been shown to impact morphogenetic processes. Indeed, in fish, as in mammals, the harmonious development of organism requires adequate levels of nutrients and particularly vitamins such as vitamin A (VA), vitamin D (VD), and vitamin C (VC) among others, which must be correctly provided by diet.

Development of Genomic Resources and Tools

A better understanding of biological processes and related environmental factors involved in the perturbation of the morphogenesis requires the availability of resources for the application of genomic approaches. It is indeed well established in many species, from drosophila to human and including fish, that embryonic and post-embryonic ontogenesis are notably underpinned by the regulation of the expression of hundreds or even thousands of genes involved in several biological processes associated with the development of organs and their functions (migration, cell proliferation and differentiation, metabolic pathways, etc.). The recent development of genomic resources (EST originated from cDNA libraries and the resulting cDNA or oligo microarrays) in various fish species of economical interest allows a large-scale investigation of gene expression in different tissues. A broad range of aquaculture species have been covered by the recent development of genomic tools (for review, see Canario et al., 2008). The availability of ESTs from European sea bass has grown significantly during the last 5 years (>50,000 EST) with the development of cDNA libraries within the frame of the European project Aquafirst and the Marine Genomic Europe (MGE) network of excellence. The interest of cDNA libraries resides in the tissue and physiological status of the organism from which the RNA samples have been extracted. Indeed, it has been shown in vertebrates (Baldessari et al., 2005), and particularly in fish (Bai et al., 2007; Kane et al., 2008), that a large part of genes involved in embryonic and post-embryonic development were absent from adult cDNA libraries. According to that, several libraries have been developed from samples, including RNA originated from embryonic or post-embryonic fish species, in order to investigate and characterize molecular regulations that occur during ontogenesis (e.g., medaka (*Oryzias latipes*), Kimura et al.,

2004; fathead minnow (*Pimephales promelas*), Kane et al., 2008; rainbow trout (*Oncorhynchus mykiss*), Govoroun et al., 2006; gilthead sea bream (*Sparus aurata*), Sarropoulou et al., 2005). In European sea bass, novel insights have been recently obtained from a cDNA library enriched in genes involved in developmental processes, since the collection of cDNA resulted from suppression subtractive hybridization (SSH) of RNA originated from larval samples with RNA obtained from juveniles (Darias et al., unpublished data).

The development of libraries provided new sequences, allowing new dedicated studies on genes implied in biological processes of interests (developed in "impact of nutritional factors" part). The availability of these large numbers of ESTs has also enabled the production of cDNA and oligo microarrays in many marine fish species. Microarrays allow simultaneous measurements of the relative expression of thousands of genes in different samples. The development of such microarray technology enabling overviews of the genes transcripts and related biological processes involved in the complexity of larval ontogenesis has been recently successfully used to investigate marine fish larval development (gilthead sea bream, Sarropoulou et al., 2005; Atlantic halibut (*Hippoglossus hippoglossus*), Douglas et al., 2008), including European sea bass (Darias et al., 2008). Such "*without a priori*" approach gives a global dimension and complements other works based on dedicated analysis of the expression of genes involved in specific biological and physiological processes during ontogenesis (i.e., myogenesis, Steinbacher et al., 2007; digestion, Srivastava et al., 2002; immune system, Corripio-Miyar et al., 2007).

Transcriptomic Investigation of European Sea Bass Larvae Development

In order to provide insights and better understand the mechanisms capable of disturbing marine fish larvae ontogenesis, it is first necessary to decipher the sequences of gene expression regulations underlying the harmonious development of organisms. The experimental rearing facilities at Ifremer laboratory allow the investigation of the developmental and physiological changes underpinning the standardized production of European sea bass juveniles of quality. On the basis of this facility, time course investigation of gene expression patterns has been performed during European sea bass larval development using microarray hybridization.

The investigation has been first performed by using heterologous hybridization of rainbow trout microarray since genomic tools were not available at that moment for European sea bass (Darias et al., 2008). This strategy was previously used for the analysis of other fish transcriptomes (Renn et al., 2004; von Schalburg et al., 2005; Cohen et al., 2007; Kassahn et al., 2007). The transcriptome was addressed at different stages related to developmental events (from 7 to 45 dph) in order to get an overview of the key genes and related biological processes involved in larval ontogenesis. This analysis allowed to evaluate the relative expression of 1278 distinct genes involved in 914 different biological processes (for more details, see Darias et al., 2008; Figure 4.2). This first investigation was next completed by a more exhaustive approach based on the use of a European sea bass cDNA microarray including more than 4000 distinct genes with functional annotation (1483 biological functions represented) developed within the frame of the European project Aquafirst and the network of excellence MGE (unpublished data). Data obtained from the abovementioned transcriptomic approach showed the complexity of ontogenic regulation occurring during larval development as revealed by the large variety of biological processes associated with regulated genes (Darias et al., 2008). Interestingly, data obtained from both experiments indicated that the biological functions represented by the genes differentially expressed throughout

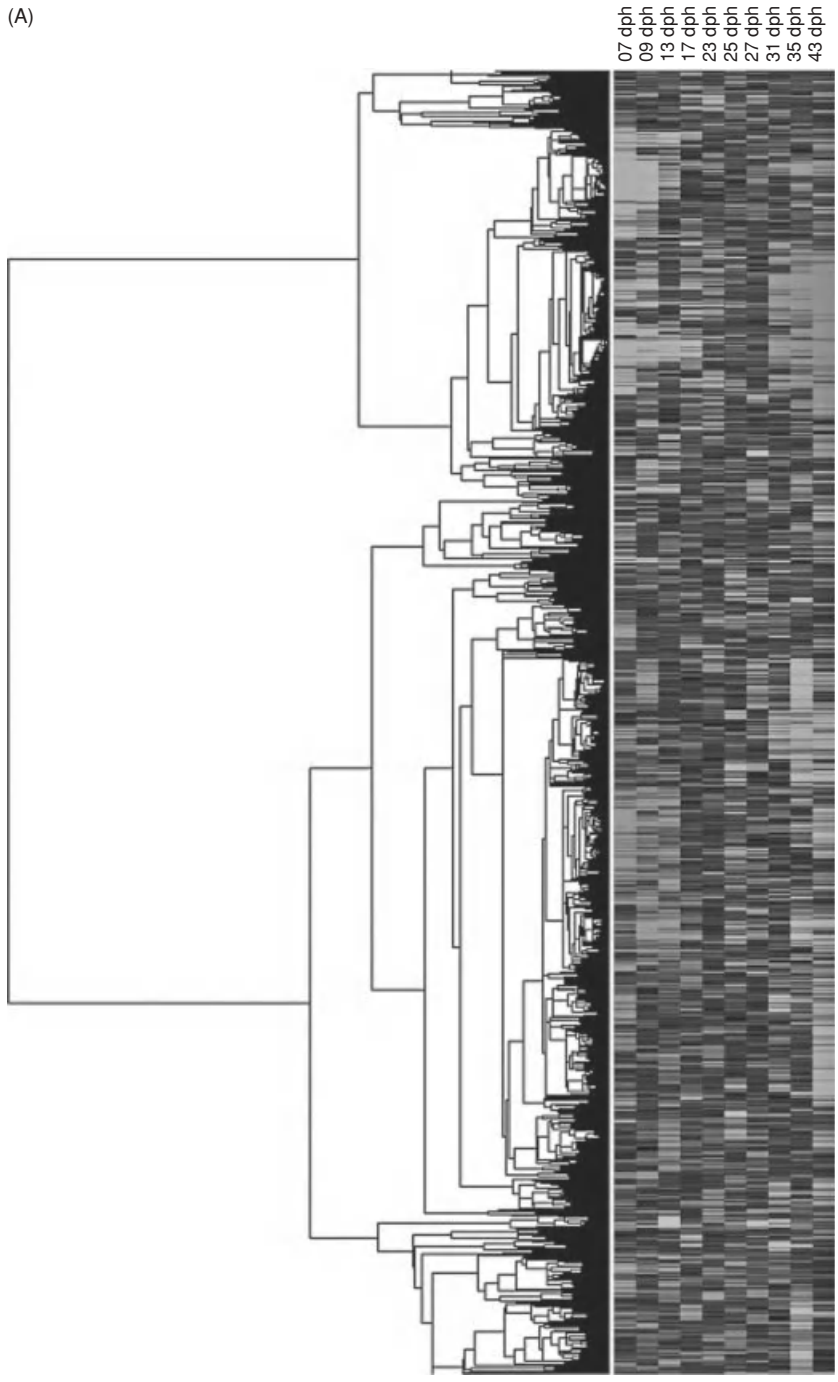

Figure 4.2 Clustering analysis of microarray studies of gene expression during early development of European sea bass. (A) Global hierarchical clustering of 1278 differentially expressed genes throughout sea bass larval development. (B) Gene clusters related to biological processes of interest. Genes are annotated with official gene names. Adapted from Darias et al. (2008.) See color insert.

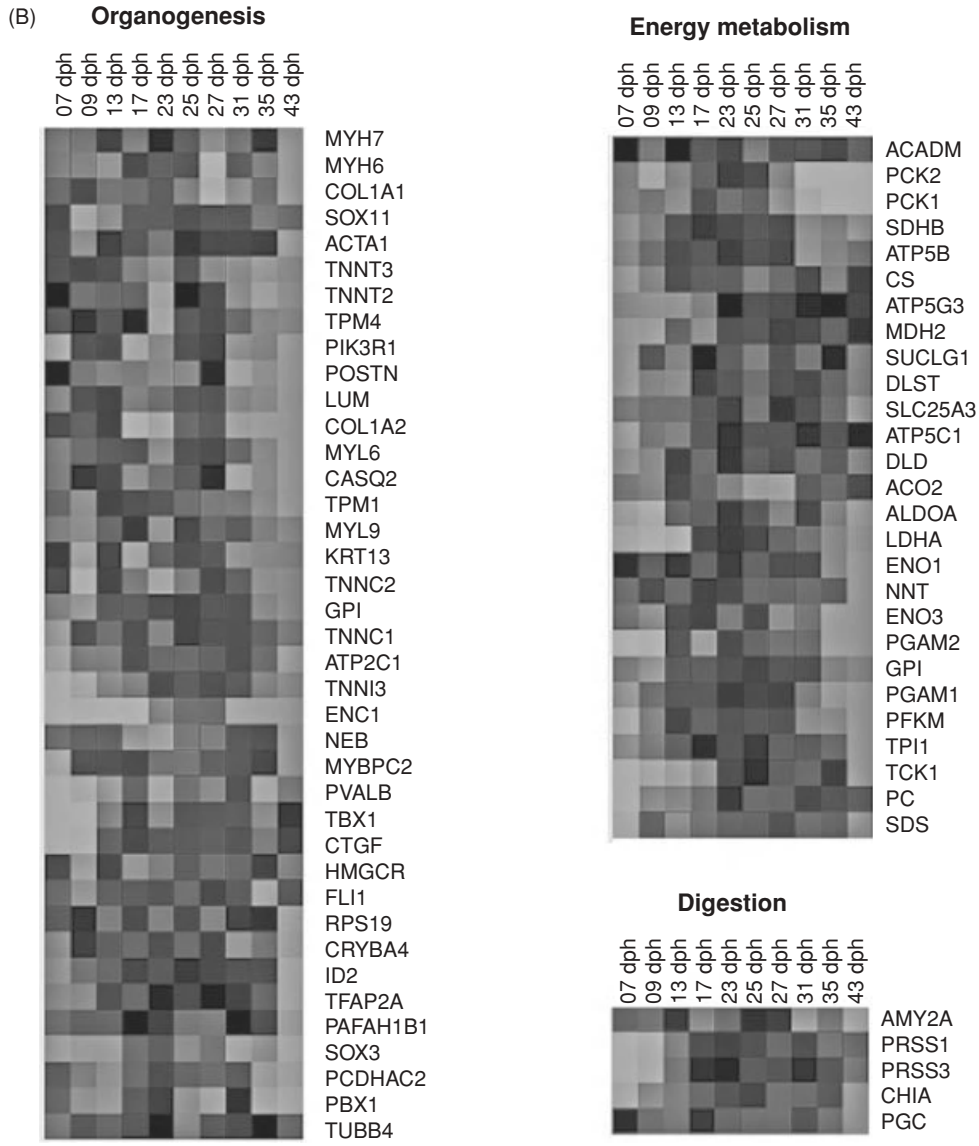

Figure 4.2 *(Continued)*

larval development are particularly related to (i) organogenesis (muscular, visual and neural development, ossification), (ii) maturation of essential functions such as the digestive function, and (iii) the regulation of metabolic pathways (Figure 4.2).

Organogenesis

As mentioned previously in this chapter, harmonious organogenesis during the post-embryonic phase is essential for larval survival. Particularly, it is crucial that larvae develop

their locomotive and visual skills for feeding and predator avoidance. Interestingly, transcriptomic analyses revealed a stimulation of the expression of genes involved in muscular development throughout the European sea bass larvae ontogenesis. Indeed, genes implicated in myogenesis (cysteine and glycine-rich protein 3, myotrophin) are shown to be significantly up-expressed from day 15 to 22 post hatching and followed the expression patterns of numerous genes involved in muscular contraction, such as titin, tropomyosin, telethonin and both light and heavy chains of myosin. Such transcriptomic data, together with other high throughput expression results obtained in gilthead sea bream (Sarropoulou et al., 2005) and Atlantic halibut (Douglas et al., 2008), explain at the molecular level the transition of myosin chains isoforms from embryonic to larval stage (Scapolo et al., 1988) during the muscular development of European sea bass larvae. Moreover, transcriptomic approach in this species also revealed that the development of the muscular tissue during larval ontogenesis was associated with the regulation of genes involved in neuromuscular transmission. *MUSK* (muscle, skeletal receptor tyrosine–protein kinase) and *KIF1B* (kinesin-like protein), involved in neuromuscular junction organization and neuromuscular synaptic transmission respectively, were up-expressed from 22 dph.

In addition, data obtained in European sea bass from both heterologous and homologous hybridizations pointed out the regulation of genes involved in visual system development during larval ontogenesis. Results indicated that transcription factor AP-2-α, required for the lens vesicle morphogenesis and genes encoding different types of crystallins (μ-crystallin homolog, β-crystalline A4), which are structural proteins found in the lens, was up-expressed during the first stage of larvae development. Such stimulation of genes implicated in visual perception is confirmed by data obtained from the SSH enriched for genes up-expressed during early larval ontogenesis, which included retinal cone rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase, γ-crystallin M2, and green-sensitive opsin (Darias et al., unpublished data). The relative high expression level of genes related to visual function during early stages of larval development can explain the fact that most of the marine fish species exhibit fully pigmented and functional eyes within the first week after hatching. Such visual development is necessary for the successful passage through the early stages since visual acuity is essential for feeding.

However, the sensory perception of environmental stimuli is not only dependent on eye formation but also on sensory neural system development. Interestingly, Darias et al. (2008) showed an up-expression of genes implicated in the onset of the visual system during the early stages of larval development concomitantly with that of genes involved in neural development: Platelet-activating factor acetylhydrolase IB subunit alpha, implicated in proliferation and migrations of neurons (Assadi et al., 2008); protocadherin-α, involved in the development of serotonergic projections (Katori et al., 2009); and *sox3*, required for both neural fate and differentiation (Dee et al., 2008). These data are in agreement with those obtained in zebrafish, describing the development of peripheral sensory structures at larval stage (Sapede et al., 2002) and post-embryonic neural proliferation in brain (Wullimann et al., 1999).

Marine fish species that undergo indirect development do not exhibit an ossified skeleton at hatching (Faustino and Power, 1998; Darias et al., 2010b). Together with muscular and neural development, the skeletogenesis process is vital for swimming performance and more generally to provide a multifunctional solid structure to the organism. Accordingly, microarray studies revealed that transcripts related to genes involved in bone development (collagens, matrix Gla protein, asporin, basement membrane-specific heparan sulfate proteoglycan core protein, periostin, and connective tissue growth factor precursor) exhibited increasing relative levels throughout European

sea bass larvae development, with a special increase around 20 dph. This finding is in total agreement with data obtained by double-staining coloration that revealed first signs of vertebral column ossification/mineralization occurring around 30 dph in European sea bass while the craniofacial skeleton ossifies earlier (10 dph; Darias et al., 2010a).

Digestive Function

Transcriptomic analysis revealed that numerous genes involved in digestive functions exhibited co-expression patterns during European sea bass larvae development. Interestingly, as mentioned earlier for muscular and nervous development, the period between 15 and 22 dph appears to be a turning point concerning the ontogenesis of digestive system in European sea bass.

Genes coding for digestive enzymes involved in carbohydrate (pancreatic α-amylase) and protein (trypsins and gastricsin precursor) digestion were strongly regulated during this period. Genes encoding basic proteolytic enzymes such as trypsin exhibited an increasing pattern of expression from 7 to 17 dph, and amylase expression decreased from 17 dph onwards.

These data revealed a progressive adaptation of digestive enzymes of larvae fed microdiets containing approximately 60% protein, which are in good agreement with results from previous works with several other fish species (Cahu and Zambonino-Infante, 1994; Srivastava et al., 2002; Murray et al., 2004; Darias et al., 2007). A better understanding of the chronology and timing of the maturation of the digestive functions is strategic in aquaculture in order to prevent as much as possible the important mortality that occurs during the transition from endogenous to exogenous feeding (Hjort, 1914) and to replace as early as possible the live preys by artificial diet. Feeding is essential for growth and survival of fish larvae, especially after yolksac resorption, since external food have to be digested and meet the nutritional needs of the organism during development. The transition from a less efficient basic protein digestion in the intestinal lumen to a more efficient acidic protein digestion, which becomes effective once the gastric glands of the stomach are completely functional, characterizes the carnivorous fish such as European sea bass (Zambonino-Infante and Cahu, 2001). 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 pattern of expression of gastricsin precursor together with trypsin and amylase profiles obtained in transcriptomic approach revealed that European 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).

Metabolic Pathways

Maturation of digestive function together with behavioral, feeding, and morphological changes occurring during larval development should be associated with global metabolic regulation. Accordingly, data from microarray analysis experiments also indicated differences in transcript levels of numerous genes involved in energy metabolic pathways throughout European sea bass larvae ontogenesis. Interestingly, the transcriptomic pattern revealed the evolution of the mode of energy production from an aerobic (tricarboxylic acid cycle, TCA) to an anaerobic one, directly related to the development of white muscular tissue and change in swimming activity. Indeed, while nearly all of the genes implicated in glycolytic pathways (glyceraldehyde 3-phosphate dehydrogenase; fructose–bisphosphate aldolase A, pyruvate kinase, α- and β-enolases, phosphoglycerate mutase 1 and 2, 6 phosphofructokinase muscle type, triose phosphate isomerase, L-lactate dehydrogenase A chain and glucose-6-phosphate

isomerase) exhibited an increasing expression pattern throughout larval development, correlated with that of genes involved in muscular development, several genes related to TCA exhibited higher amounts of transcripts within the early stage of development (day 7–17 post hatching; Darias et al., 2008). These transcriptomic results are in agreement with previous data obtained by biochemical approach, indicating an energy metabolism almost entirely aerobic in embryos and early larvae (Wieser, 1995) and an increase of the anaerobic power in the fast muscle fibers during the transformation from larval to juvenile stage.

In summary, the high number of genes and the related biological processes regulated during the larval development, revealed by transcriptomic analyses, shows the complexity of the molecular mechanisms underlying post-embryonic ontogenesis in marine fish species. Even if such transcriptomic approaches are based on the investigation of gene expression in pool of whole larvae, which is prejudicial to analyze tissue-specific regulation of genes (Darias et al., 2008; Power et al., 2008), the major interest is to provide insights into the complex molecular networks underlying the maturation of marine fish larvae and particularly to allow the observation of coordinated expression of genes that function in common or different biological processes. Co-expression patterns of genes involved in different biological or molecular processes confirm that the development of organisms results from the interaction of different regulatory pathways and not just a sum of independent molecular mechanisms.

These complex molecular and cellular mechanisms are capable to buffer organisms from expected environmental cues (Hamdoun and Epel, 2007). However, these adaptive capacities exhibit physiological limits since the development of larvae can be affected by environmental and nutritional factors. Considering the role of the transcriptome in the regulation of biological processes and the crucial role that nutrition plays in the development of marine fish larvae, special attention has been paid throughout the last five years to better understand the impact of nutritional factors on the expression of genes involved in the development of marine fish species.

Impact of Nutritional Factors

In order to decrease production costs as well as to sustain production of high and constant quality juveniles, larviculture has required compound diet substitution for live prey (traditionally, rotifers and *Artemia*). However, the formulation of compound diets adequate for fish larval development is not easy to achieve since nutritional requirements during development are not well documented. Diet formulations were first based on data obtained from juveniles that hardly met the specific requirements during larvae development. During the last decade, several works have been performed in European sea bass in order to better define the optimal contents of nutrients in larval diets based on phenotype determination including morphological analysis and gene expression patterns in fish species of economic interest, especially within the frame of a European project (Finefish, Sixth Framework Programme, European Union). Special attention has been paid to determine the impact of dietary vitamin contents. In vertebrates, including fish, it is recognized for several decades that vitamins influence the biological processes underpinning embryonic and post-embryonic development and particularly morphogenesis (Hurley, 1967; Giroud, 1968). The known influence of vitamins on the appearance of malformations during fish larval ontogenesis has encouraged researchers to investigate the molecular actors and related mechanisms underlying the skeletogenesis process as well as to determine more accurately the dietary vitamin levels inducing adequate larval morphogenesis. These efforts aimed to solve skeletal malformations, one of the most important bottlenecks in larviculture leading to severe economic losses for the aquaculture industry.

Impact of Vitamin A

Vitamin A (VA) is a fat-soluble nutrient, essential in vertebrates throughout embryogenesis and post-natal development. As fishes are not able to synthesize VA, they have to take it from the diet, in form of carotenoids from vegetal and/or as retinyl esters, such as retinyl palmitate, or as xanthines from animal sources. However, those provitamin A metabolites need to be transformed to its active form, mainly to retinoic acid (RA), through a complex transport and enzymatic system that reflects that fish needs to control tightly the homeostasis of this essential nutrient (reviewed by Rodrigues et al., 2004).

Among retinoids (VA metabolites), the main active forms are 11-*cis*-retinal, all-*trans*-retinoic acid (atRA), and 9-*cis*-retinoic acid (9cRA). 11-*cis*-retinal serves as the chromophore of the visual pigments in retina (Pepe, 1999); whereas both isomers of RA regulate the nonvisual function of VA, controlling cell proliferation and differentiation in tissues (Ross et al., 2000). Interestingly, a recent study has shown that RA degradation products (4-oxo-RA, 4-OH-RA, and 5,6-epoxy-RA) originated from cytochrome P450 26A1 Cyp26A1 enzymes could also regulate gene transcription and were able to fully rescue the VA deficiency (Reijntjes et al., 2005).

The main pathway by which RA isomers controls gene expression is through the activation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which present three different isoforms (α, β, and γ) (Germain et al., 2006a, 2006b). However, while atRA is only able to bind to RARs, 9cRA can transcriptionally activate both kind of nuclear retinoid receptors (Germain et al., 2006a, 2006b). By the specific binding of RA isomers to the RAR and RXR, they form homodimers, such as RXR/RXR, or heterodimers (with other nuclear receptors such as THR, VDR, and PPARγ) that bind to specific nucleotide sequences (retinoic acid response elements, RAREs) in the promoter region of a large number of genes (Balmer and Blomhoff, 2002). By direct gene expression regulation through the exhibition of a RARE in the gene promoter or through downstream signaling cascade, RA could control directly or indirectly the transcription of more than 300 genes (Balmer and Blomhoff, 2005).

Despite the enormous progress in fish genomic-based tools (Ortiz-Delgado et al., 2006; Canario et al., 2008; Darias et al., 2008; Ferraresso et al., 2010), gene expression studies regarding RA transcriptional regulation in fishes are still limited. In that sense, several works have been focused on zebrafish as a lower vertebrate-species model (Hale et al., 2006; Tallafuss et al., 2006; Waxman and Yelon, 2007), whereas some others in aquaculture marine fish species (Haga et al., 2002a, 2002b, 2003; Villeneuve et al., 2004, 2005, 2006; Fernández et al., 2008, 2009; Mazurais et al., 2009).

As European sea bass accept compound diets from first feeding (Cahu et al., 2003), and those diets present a more stable nutritional composition than enriched live preys (Gimenez et al., 2007), this species has become a suitable marine fish species to point out the critical role of VA in marine fish morphogenesis, and consequently, a large amount of research has been focused on this topic (Villeneuve et al., 2004, 2005, 2006; Darias et al., 2008, 2010a; Mazurais et al., 2008, 2009; Geay et al., 2009; Georga et al., 2011).

VA function and molecular mechanism studies in European sea bass started with the description of the gene expression pattern of the retinoid receptors throughout the larval development by relative gene expression and RNA/RNA hybridizations and qPCR of some RARs (RARα, RARγ, and RXRα; Villeneuve et al., 2004). The former authors showed that gene expression of RAR isoforms increased, whereas RXRα expression decreased between 10 and 42 dph. Furthermore, regarding tissue domains, Villeneuve et al. (2004) found that RARα was expressed in jaws at 5 dph and

in jaws, vertebrae, liver, musculature, pectoral fins, brain, neural tissues, eyes, and branchial arches at 37 dph, reflecting the critical role that RARα plays in the morphogenesis of many tissues. In contrast, more restricted expression was found for RARγ at the same developmental times, while RXRα expression was only localized in the exocrine pancreas, liver, thyroid, kidney, heart, jaws, and teeth. These results suggested that each retinoid receptor played a specific role in proliferating and differentiating tissues and that disruption of the retinoid signaling process could be a causative factor of the abnormal skeletogenesis and morphogenesis in European sea bass. Those hypotheses were confirmed later on when Villeneuve et al. (2005) found a linear correlation between VA level and percentage of deformed individuals. Furthermore, authors found that high incidences of skeletal deformities were mainly associated with an upregulation of RARγ expression in the highest VA dose group during the first 2 weeks after hatching. In the same study, an increase of RARα expression and a decrease of RXRα expression were also found in fish fed high VA doses compared with those fed a control diet. Interestingly, Villeneuve et al. (2006) showed that the effect of dietary VA level depend on the larval developmental stage, sea bass being particularly affected in its development by dietary VA content before 13 dph. In that study, skeletal deformities in fish fed hypervitaminosis A were correlated with the high expression of RARγ and bone morphogenetic protein 4 (BMP-4) during early development, and an increase of RXRα expression in fish fed high VA doses at later developmental phases (Villeneuve et al., 2006). However, it should not be neglected the possibility that an expression disruption in other genes, particularly other retinoid receptor isoforms, could take place and affect the normal harmonious development of larvae. Interestingly, recent works show that VA dietary content modulates the expression of particular genes that seems to not only cause the appearance of specific types of skeletal deformities (Mazurais et al., 2009) but also alter the skeletal shape of normal fish in sea bass (Georga et al., 2010). Mazurais et al. (2009) demonstrates that excess of dietary VA levels cause disruption in gene expression, which leads to the appearance of skeletal deformities, and that suboptimal dietary VA content implied a lower *Hoxd*-9 gene expression, which could explain why fish fed VA-deficient diet presented a partial or complete lack of pelvic fin. That research work highlighted the gene expression regulation of VA on *Hox* genes family as have been reported previously in other marine fish species (Suzuki et al., 1999). Also, it has been reported that high dietary VA content reduces sensitively gene expression of endothelin-1 during early European sea bass development (10–15 dph), which may be involved in the development of the craniofacial deformities observed in those fishes (Geay et al., 2009). In addition to the disruption of some genes related to skeletal structures by nutritional VA imbalances, it has been shown that RA strongly induces premature myoD expression in the presomitic mesoderm of zebrafish affecting fast muscle differentiation (Hamade et al., 2006). Since muscle and skeleton develop from the same precursor tissue, the embryonic mesoderm, an imbalance of the muscle tissue development, could also lead to the appearance of skeletal deformities. However, further works have to be done to point out the crosstalk between muscle and bone (mechanostat theory, Frost, 1987) and its critical role on fish morphogenesis.

Regarding VA function and nutritional imbalance effects on larval development, it should be noted that although several studies have pointed out the key role of VA in fish morphogenesis and skeletogenesis through gene expression regulation, further work is needed to decipher the underlying molecular mechanism by which suboptimal dietary VA content disrupts gene expression and compromises marine fish larval development and performance. The impact of dietary VA on marine fish development and aquaculture production will be determined by the chemical form

and dietary dose in which VA is supplied, the duration of this supply, the fish developmental stage, and the particular ontogeny of the fish species (Fernández and Gisbert, 2011). Therefore, dietary VA requirements need to be fine-tuned for each fish species.

Impact of Vitamin D

Vitamin D (VD) is a fat-soluble secosteroid that is especially crucial for maintaining calcium and phosphate homeostasis and protecting skeletal integrity (DeLuca, 2004). Contrary to mammals, which can synthesize VD from a cholesterol derivative through photosynthesis in the skin, under ultraviolet B radiation exposure, fish cannot synthesize VD from solar energy because all traces of irradiation are absorbed by sea water in the first few meters of depth (Lock et al., 2010). Copping (1934) was the first to demonstrate that the zooplankton, which is at the basis of aquatic food chains, was rich in VD and then could be the origin of VD supply for fish in natural environment. Even if two forms of VD (vitamin D_2 (VD_2) and vitamin D_3 (VD_3)) have been detected in high concentration in plankton, VD_3 is almost the primary storage form in fish (Mattila et al., 1997), especially because of both higher bioavailability and binding to transport protein (Lock et al., 2010). After ingestion, VD3 is transported via VD-binding protein in liver where it is hydroxylated to 25-hydroxyvitamin D_3. 25-hydroxyvitamin D_3 is next hydroxylated to form the most active metabolites 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and to a lesser extent 24,25-dihydroxyvitamin D3 [$24,25(OH)_2D_3$] in liver and other tissues including kidney (Takeuchi, 1994; Sundh et al., 2007) even if more metabolites of VD_3 with physiological significance may exist in fish (Lock et al., 2010). Despite the putative existence of membrane-binding protein for $1,25(OH)_2D_3$ in fish, most of VD action described until now in fish is mediated through binding to nuclear $1,25(OH)_2D_3$ receptor (VDR). After binding to VDR, which belongs to the nuclear receptor superfamily of steroid/thyroid hormone receptors, the hormone/receptor complex forms a heterodimer with RXR (as mentioned in Section "Impact of Vitamin A") and is able to regulate the expression of genes by interacting with specific DNA sequence (vitamin D responsive element) in their promoter (Lock et al., 2010). In mammals, $1,25(OH)_2D_3$ has been shown to regulate genes involved in calcium binding and transport in the intestine, resulting in stimulation of active calcium uptake and bone mineralization (Christakos et al., 2003). VD has also been shown to act directly on osteoblasts to inhibit proliferation, modulate differentiation, and regulate mineralization of the extracellular matrix in mammals (Sutton et al., 2005). Furthermore, it is now recognized that VD regulates numerous genes involved in a wide range of biological processes, including cell proliferation and differentiation (Samuel and Sitrin, 2008).

Similarly, VDR expression pattern detected in a wide range of tissues in fish (Lock et al., 2007; Craig et al., 2008) reflects that VD plays, as in mammals, an important role in several biological processes. Indeed, numerous works confirm actions of VD in intestine, gills, kidney, and bone of fish (for review: Lock et al., 2010). Moreover, expression of VDR detected during early development of fish also revealed the implication of VD in ontogenic process (Fleming et al., 2005).

As fish obtain VD through the food chain, it is necessary to supply adequate dietary VD concentrations for breeding fish along the life cycle and particularly during larval development. Only few studies exist on the impact of VD on the molecular pathways involved in fish larval morphogenesis. Recently, the impact of different dietary VD (provided through VD_3 form) levels on ossification and muscular development processes were investigated in European sea bass based on morphologic analysis and dedicated gene expression analysis

(Darias et al., 2010b; Alami-Durante et al., 2011).

On the basis of the expression of BMP-4, osteocalcin, VDR, and transient receptor potential cation channel subfamily V member 6 (TRPV6), Darias et al. (2010b) suggested that the suitable VD level for optimal European sea bass larval development is within a restricted range with slight variations inducing a cascade of severe physiological disruptions likely resulting in skeletal deformities. The expression of BMP-4, which is involved in the achievement of the mature phenotype of osteoblasts, has been first shown to decrease with the descent of dietary VD levels. This downregulation of BMP-4 expression in larvae fed with low VD could negatively influence the availability of potentially osteoblastic cells leading to an ossification delay. In the same work, VD deficiency has also been shown to disrupt mineralization process through (i) regulation of calcium absorption at intestinal level as revealed by reduced TRPV6 expression and (ii) direct action on bone tissue through downregulation of osteocalcin gene expression. TRPV6 is indeed considered as the major transcellular mediator of calcium uptake from the intestinal lumen (Hoenderop et al., 2005), and osteocalcin is one of the marker genes for the progression of osteoblastic differentiation being associated with mineralization of the extracellular matrix (Lian and Stein, 1995). All together, these transcriptional regulations of osteoblastic cells differentiation and bone mineralization, in addition to the observed malformation rate and delay of growth, revealed that a VD level of 19.2 IU/g diet appeared necessary to obtain an harmonious larval growth and morphogenesis in European sea bass larvae. This result is in agreement with expression data obtained by Alami-Durante et al. (2011), which revealed that VD dietary level lower than 19.2 IU/g diet induced a decrease of the expression of myogenic regulatory factors (Myf5, MyoD, and myogenin) involved in the recruitment and the growth dynamics of skeletal muscle fibers at both 22 dph and 44 dph and associated with lower body weight of the larvae at 45 dph.

Taken together, these data confirm the regulation by VD of different processes involved in ontogenesis of fish at larval stage. This idea was also recently confirmed in the laboratory by transcriptomic investigation of genes regulated by VD deficiency during European sea bass larval development (unpublished data).

Impact of Vitamin C

Ascorbic acid (AA) is a water-soluble vitamin widely known for its powerful antioxidant properties, protecting low-density lipoproteins from oxidation, reducing harmful oxidants in the stomach, and promoting iron absorption (Padayatty and Levine, 2001). Apart from these nonenzymatic actions, AA also acts as a cosubstrate for hydroxylase and oxygenase enzymes involved in the biosynthesis of procollagen, carnitine, and neurotransmitters (Tolbert, 1979). This vitamin is necessary for the formation of collagen and cartilage, as well as bone formation and remodeling (Wilson and Poe, 1973; Kraus et al., 2004). AA can be synthesized de novo from D-glucose and D-galactose in many vertebrates, including some groups of fishes that have retained ancestral characters (e.g. acipenserids), contrary to teleost fish, including European sea bass, which are unable to synthesize AA because of the lack of the last enzyme of the biosynthetic pathway (L-gulonolactone oxidase; Dabrowski, 1990; Dabrowski, 1994; Maeland and Waagbo, 1998).

Similar to the situation of VD, the fact that some fish species are not able to synthesize AA turns indispensable the knowledge of dietary requirements for this vitamin to assure adequate growth and welfare. In this respect, some nutritional studies have been performed in fish species, revealing the key role of AA for the correct development of the skeleton (for review, Halver et al., 1974; Merchie et al., 1997). In mammals, the action of this vitamin

as a cofactor of prolyl and lysyl hydroxylases, which catalyze the hydroxylation of proline and lysine, respectively, is critical for stabilizing the helical structure of collagen, the most abundant protein in the extracellular matrix of cartilage and bone (Kern et al., 2001). In fish species, including European sea bass, severe deformities induced by AA deficiency have been shown to be associated with the impaired collagen and support cartilage formation in most tissues (Halver et al., 1974; Terova et al., 1998).

Although the direct effect of AA deficiency in the development of skeletal deformities is well demonstrated in marine fish species (Merchie et al., 1995), the mechanisms involved in this process are poorly investigated, especially during skeletogenesis. On the basis of the sequences available in databases, our recent work investigated the impact of dietary AA on the expression of genes involved in different ontogenic processes and particularly those related to ossification in European sea bass larvae (Darias et al., unpublished data). Larvae fed diets with low AA content (<50 mg AA/kg) showed higher RARγ expression than the other groups in concomitance with lower levels of osteocalcin expression, suggesting a delay in skeletal development. The high expression levels of RARγ, which is preferentially co-localized to the precartilage condensation and cartilage (Cash et al., 1997), were also associated with high amounts of PPARγ transcripts, which promote adipocyte differentiation (Casteilla et al., 2007), suggesting reduction of osteoblast formation in larvae fed low AA level. Higher levels of both PPARγ and RARγ transcripts combined with lower levels of osteocalcin mRNA have previously been shown to be associated with skeletal deformities in European sea bass larvae (Villeneuve et al., 2006; Mazurais et al., 2008). The impact of dietary AA on osteoblast determination and differentiation in this species confirmed previous data obtained in mammals (Carinci et al., 2005). The mechanism by which AA sustains pre-osteoblast proliferation and commitment is known to be mediated through the synthesis of collagen type I, interaction with α2- and β1-integrin, activation of the mitogen-activated protein kinase pathway, and phosphorylation of osteoblast-specific transcription factors (Carinci et al., 2005). BMP-4, a potent osteoblast differentiation factor essential for osteoblasts to achieve their mature phenotype (Abzhanov et al., 2007), was also investigated in European sea bass. As mentioned previously, the influence of this gene on European sea bass larval morphogenesis takes place during the early stage of development, and its relative abundance in whole larvae decrease with larval growth (Darias et al., 2010b). Authors suggested that this decrease of relative BMP-4 messenger level during development could be an indicator of the osteoblast mature phenotype achievement. Interestingly, recent studies have shown that BMP-4 transcript levels were significantly higher in larval groups fed diets with low AA content at day 15 post hatching, supporting a negative regulation of ostebolast maturation in those dietary groups. The impact of AA on osteoblast proliferation was also tackled during European sea bass larval development through the evaluation of IGF1 expression, which is known to increase with osteoblasts proliferation and to play a major role in stimulating mature osteoblast function (Hughes et al., 2006). Interestingly, these studies demonstrate that European sea bass larvae fed diet with low AA content displayed low levels of IGF-1 mRNA levels, revealing an impairment of osteoblast maturation. In addition to impacts of AA on biological pathways related to osteoblast proliferation and/or differentiation, attention was also paid to other biological processes related to ossification, such as mineralization. It has to be pointed out that recent data showed, in European sea bass larvae, a high correlation between the gene expression profile of SVCT-1 (sodium-dependent vitamin C transporter 1, which is the most important AA transporter in intestinal cell) and osteocalcin on one hand and the amount of cartilaginous tissue and bone mineralization degree on the

other. Moreover, it has been observed that this profile was inversely correlated with the incidence of skeletal deformities, suggesting a possible disruption of the mineralization process. Expression data obtained through dedicated qPCR approaches globally revealed a key role played by dietary AA content on the osteoblast differentiation or proliferation processes as well as on the different pathways involved in mineralization.

In summary, these studies related to molecular effects of vitamin A, D, and C indicate that vitamins have several processes, such as ossification/mineralization, as common targets and that they likely interact to regulate biological pathways during fish larval development as it has been shown in adult (Lock et al., 2010). Interaction between VA and VD could easily been related to the heterodimerization of their receptor, which form a RXR/VDR complex since both vitamins likely interact through activation and transactivation of heterodimer. As mentioned previously, it has also been shown that diet with low AA content induced higher RARγ expression in European sea bass larvae, suggesting interaction between VC and VA pathways. Similarly, Darias et al. (unpublished data) recently demonstrated that AA could influence the expression of VDR during sea bass larval development reinforcing an interaction between VD and VC pathways. Finally, such interactions between dietary vitamins indicate that each vitamin requirement has to be determined using integrated approach.

Conclusions

Expression data obtained from high throughput or dedicated approaches have given precious insights into understanding the biology of early life stages in European sea bass as a model for marine pelagic fish species. The transcriptomic approaches, based on heterologous or homologous hybridization of cDNA microarrays, have been successfully used to reveal differentially expressed genes that are essential for the harmonious fish development. These results can be paralleled and explained the substantial changes that occur in larval morphology, metabolism, and behavior to produce organisms capable of surviving in post-embryonic environments. These first transcriptomic approaches also constitute a basis for future investigation of marine fish larval ontogenesis and open up possibilities for studies focusing on annotated (or not) genes with interesting expression patterns. However, these data have to be completed in the near future by new experiments based on the use of new methodologies such as microdissection, which will enable the analysis of transcriptome in discrete tissue of larvae, thus revealing tissue-specific regulation of gene expression. RNA originated from microdissected samples will be ideally hybridized on oligo microarray including still growing number of annotated probes; oligo microarray support allows investigation of splicing variant and genes having similar cDNA sequences but different functions that belong to the same family. Transcriptomic data will also be acquired using new high throughput cDNA sequencing technologies (Illumina, 454), particularly in species for which libraries and microarray have not been developed. Finally, considering that ontogenic processes also depend on post-transcriptomic regulation, the acquisition of new annotated sequences should also profit with the proteomics approach in marine fish species, which is confronted with the problem of annotation of peptides of interest.

In summary, these optimizations will allow obtaining more precise and exhaustive information concerning genes and associated physiological processes involved in larval ontogenesis of marine fish species, and that could be impacted by atrophic and trophic changes. Data obtained should, thus, contribute to improve aquaculture practices and hence production. Holistic approaches will be particularly useful to better understand the complex physiological impact of nutrients such as vitamins on ontogenic processes. Indeed,

expression data obtained during European sea bass larval development by dedicated approach revealed that small variations of dietary vitamin contents could induce severe physiological disruptions in cascade; this include digestive tract maturation, ossification process as well as muscular development, resulting in the appearance of skeletal deformities, delayed growth, and mortality. A global understanding of biological processes involved in those regulations will contribute to better define the nutritional requirement of larvae during the post-embryonic development.

Acknowledgments

This scientific writing resulted from collaboration of Ifremer, Fish nutrition laboratory in France, and IRTA, Unitat de Cultius Experimentals, Spain. This collaboration is supported by EGIDE. Some of the work reported was carried out within the frame of FINEFISH, a collective research project of the Sixth Framework Programme of the European Union (contract 012451). IF was supported by a predoctoral MEC fellowship (reference BES-2006-12650, Spain).

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Chapter 5
Transcriptomics of the Compensatory Growth in European Sea Bass *Dicentrarchus labrax*

Genciana Terova, Samuela Cora, Tiziano Verri, Rosalba Gornati, Giovanni Bernardini, and Marco Saroglia

Abstract: The exceptionally fast growth that fish experience after periods of fasting has been called "compensatory growth" or "catch up" growth. This phenomenon, reported in a wide range of fish species, has been studied in intensive aquaculture as a means of enhancing feed conversion efficiency, but the mechanisms implicated are complex and not yet fully understood. Our research focuses on identifying candidate genes whose expression contributes to the compensatory growth induced by refeeding in sea bass (*Dicentrarchus labrax*).

As an alternative to genome sequencing for discovering genes in this teleost, we have successfully implemented a process for identifying unknown genes, assuming that a computational database resource contains orthologous sequences from phylogenetically related species. Following this similarity-driven molecular cloning and sequencing, we isolated several full-length sea bass cDNAs that encode for some physiologically relevant proteins in this species.

We have then made use of the DNA sequence data to understand the function of these genes with the aim to identify valuable molecular biomarkers influenced, either positively or negatively, by dietary manipulations. Accordingly, in this chapter, we describe the utilization of molecular cloning and functional genomics in aquacultured sea bass to generate "transcriptome-focused" information, which may enable a better understanding of the transcriptional programs that underlie compensatory growth induced by refeeding.

Introduction

In natural aquatic systems, many fish species experience periods of fasting during their life cycle because of the high variation in food availability. Although fish are highly tolerant to relatively long periods of food deprivation, energy storage and growth rate is reduced in those that experience different degrees of starvation, redirecting this energy toward maintenance metabolism (Navarro and Gutiérrez, 1995). Several changes in the variables related to growth are then displayed: decreased condition factor (an indicator of fish body shape), lipid content, muscle, and organ weight. When abundant food is encountered after a period of "nutritional stress," these fish show exceptionally fast growth rate compared with those that had uninterrupted access to food during the

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

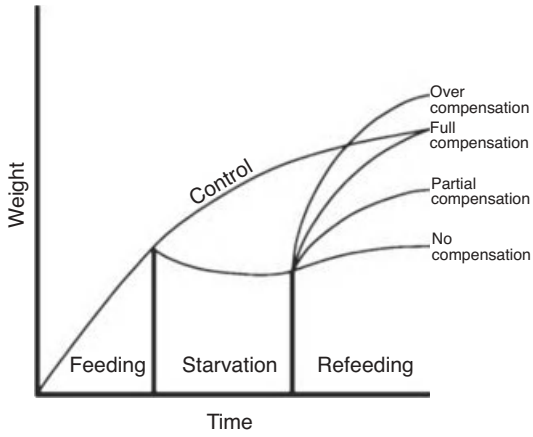

Figure 5.1 Idealized patterns of growth compensation. (From Ali et al., 2003.)

same period (Figure 5.1). This phenomenon, known as "compensatory (catch-up) growth" (MacKenzie et al., 1998), has been reported in a wide range of animals, including various fish species (Jobling et al., 1994; Ali et al., 2003; Nikki et al., 2004), but the mechanisms implicated in such rapid recovery from fasting are still not fully understood.

In particular, we know very little about the response of genes at the transcriptional level to starvation and refeeding, although this knowledge could supply greater insight into critical biochemical pathways that control vertebrate muscle growth and is also relevant to understanding the mechanisms by which the expression of such genes is regulated.

Our research focuses on identifying candidate genes whose expression contributes to the compensatory growth induced by refeeding in sea bass (*Dicentrarchus labrax*; Terova et al., 2006, 2007a, 2007b, 2008, 2009). This species is of great interest for Mediterranean aquaculture as it is an excellent food fish with high commercial value. Moreover, it also represents a good model organism for muscle growth studies as, unlike traditional fish models such as zebrafish and mekada, it shows extensive postlarval muscle hyperplasia, which contributes to its large adult size.

Accordingly, in our recent studies, we have isolated the complete cDNA sequences coding for some physiologically relevant proteins in this teleost, and then have assessed the impact of chronic feed deprivation and subsequent refeeding on their mRNA levels in different tissues, with the aim to relate these expression levels to fish feeding status.

Key Players in Fatty Acid Metabolism

European aquaculture, particularly of marine fish, is dependent upon feed grade fisheries to provide fish meal and oils traditionally used as the main protein and lipid sources in the feeds. The general trend to increase lipid content in aquafeeds for marine fish to improve growth, feed conversion, and protein utilization has produced an increase in the demand for fish oil in aquaculture. However, the capacity of the fisheries to cope with the increasing demand for fish oil has reached the limit of sustainability, owing to problems such as overfishing, climate alterations, and increasing demand from other sectors (Sargent et al., 2002). Thus, alternative oils are needed to supply lipids for aquafeeds, and those from plant seeds such as linseed, rapeseed, or soybean oils represent good candidates. Among the teleosts, freshwater species differ from marine species in their fatty acid requirements. The former have a recognized capacity to desaturate and elongate the fatty acids with 18 carbon atoms, linoleic (18:2 *n*-6) and linolenic (18:3 *n*-3) acids, into highly unsaturated fatty acids (HUFA) with 20 and 22 carbon atoms such as arachidonic (AA, 20:4 *n*-6), eicosapentaenoic (EPA, 20:5 *n*-3), and docosahexaenoic (DHA, 22:6 *n*-3) acids (Sargent et al., 2002). The HUFA play a major role in eicosanoid production (AA and EPA), vision (DHA), brain development (DHA), and regulation of expression of several genes involved in lipid metabolism (Forman et al., 1997).

In contrast to freshwater fish, marine species are assumed to have a deficient capacity to bioconvert 18 carbon precursors into HUFA and,

hence, require preformed HUFA in their diet. Although long recognized, the reasons for this deficiency in marine fish remain unclear. On the one hand, it could be because of a gene loss corresponding to an adaptation to the *n*-3 HUFA-rich marine food web (Sargent et al., 1995). On the other hand, marine fish show a deficiency in desaturase enzymes, probably because of repressed desaturase activity associated with high levels of HUFA usually present in the diet, as previously seen in mammals.

By determining the essential fatty acid requirements in marine fish and understanding the molecular basis of HUFA biosynthesis and how it is regulated in fish, the use of vegetable oils can be manipulated and optimized in aquaculture. Replacing fish oil with vegetable oils in marine fish diets has been well studied: not only fish health can be compromised by including certain vegetable oils, the nutritional quality of the flesh can also be affected by modifying muscle fatty acid profiles, which includes a reducing *n*-3 HUFA, and particularly EPA (Izquierdo et al., 2003) and altering the relationship between *n*-3 and *n*-6 fatty acids. Moreover, vegetable oils can affect the flesh quality of aquacultured fish in terms of *n*-3 HUFA source for human consumption. To avoid these problems, we need to know how the different vegetable oil diets influence fish fatty acid biosynthesis.

In recent years, significant progress has been made in characterizing fatty acid desaturases involved in HUFA synthesis. These enzymes introduce a double bond in a specific position of long-chain fatty acids and are conserved across kingdoms. Δ6 fatty acid desaturase has been shown to be the enzyme responsible for the first and rate-limiting step in the biosynthesis of HUFA from C18 PUFA (Figure 5.2), and it has been isolated from several fish species, including zebrafish, *Danio rerio* (Hastings et al., 2001); common carp (accession no. AF309557); gilthead sea bream (Seiliez et al., 2003); Atlantic cod (accession no. DQ054840); rainbow trout (accession no. NM_001124287), and Atlantic salmon (Zheng et al., 2009).

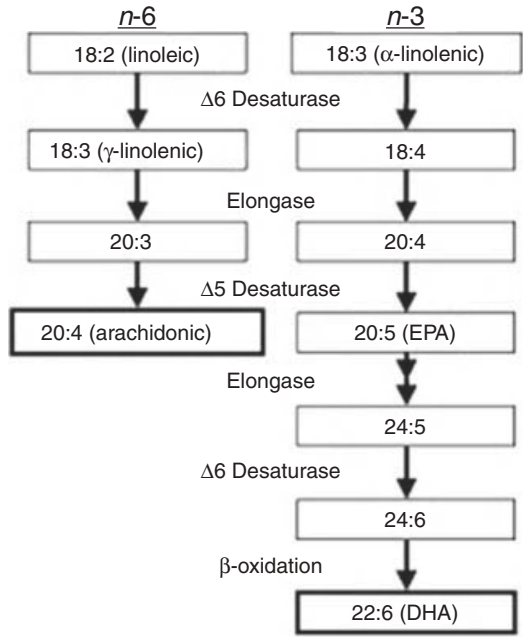

Figure 5.2 The actual pathways for *n-6* and *n-3* HUFA biosynthesis.

Many reports have described the cloning, tissue distribution, and the nutritional regulation of Δ6 desaturase in fish. A study in rainbow trout demonstrated that levels of Δ6 desaturase transcripts were nutritionally modulated in fish fed diets with varying sources of dietary lipids (Seiliez et al., 2001). Inhibition of desaturase gene expression by dietary HUFA was also shown in gilthead sea bream (Seiliez et al., 2003): Δ6 desaturase transcripts appear to be depressed in fish fed a HUFA-rich diet compared to fish fed a HUFA-free diet, indicating a nutritional regulation similar to that reported for mammalian desaturases (Cho et al., 1999). These findings are confirmed by the study of Izquierdo et al. (2008), showing that very high contents of linseed oil in gilthead sea bream diet inhibited desaturase activity, whereas diets formulated with soybean or rapeseed oils increased the relative expression of this enzyme. However, to our knowledge, there is little information regarding the response of Δ6

desaturase at the mRNA level to starvation and subsequently refeeding in marine fish.

Key Players in Intestinal Oligopeptides Transport

Digested dietary proteins in both teleosts and mammals are subject to hydrolysis by a range of proteases and peptidases that generate a mixture of free amino acids and small peptides that are efficiently absorbed across the apical membranes of enterocytes (Clements and Raubenheimer, 2006). Cellular transport of amino acids and small peptides is thus a key final step in their assimilation by the intestine. Two distinct types of brush border membrane-associated transport systems are involved in this process: (1) amino acid transport that may or may not be sodium dependent and (2) transport of small peptides (2–3 amino acids) coupled to a H^+ gradient. Intestinal peptide transport is of major nutritional significance in that the intraluminal products of protein digestion are predominantly di- and tripeptides, not amino acids as was widely believed over 30 years ago (Adibi, 2003). In addition, many amino acids are more rapidly and efficiently absorbed in peptide form (Gilbert et al., 2008a). Furthermore, the high activity of intracellular peptidases showing strict specificity for the hydrolysis of di- and tripeptides suggests that di- and tripeptides peptides, but not larger ones, may be absorbed in intact form and that free amino acids are then released intracellularly (Gonçalves et al., 2007). However, the extent to which a di- or tripeptide that is released during digestion is finally broken down at the brush border membrane or is taken up into the cell and hydrolyzed there is not known.

Peptide transport has also been well demonstrated in teleosts, where it occurs via carrier-mediated mechanisms and is highly stimulated by an inside-negative transmembrane electric potential (Thamotharan et al., 1996; Maffia et al., 1997). Proton/glycyl-sarcosine (Gly-Sar) cotransport has been described in the brush border membrane of the absorbing cells in tilapia *Oreochromis mossambicus* intestine and rockfish *Sebastes caurinus* intestine and pyloric caeca (Thamotharan et al., 1996). Moreover, H^+/D-phenyl-L-alanine cotransport has been described in eel *Anguilla anguilla* intestinal brush border membrane (Verri et al., 1992, 2000; Maffia et al., 1997).

Although results from many studies demonstrated the uptake of peptides into intestinal cells, the transporter responsible for this, designated PepT1, was not identified until 1994: Fei et al. (1994) cloned it by microinjecting mRNA isolated from rabbit intestine into *Xenopus* oocytes, which resulted in functional expression of the protein.

Oligopeptide transporter 1 (PepT1) is an integral plasma membrane protein responsible for the uptake of dietary di- and tripeptides in cells. It transports peptides against a concentration gradient by coupling the movement of substrate across the membrane, with the movement of protons down an inwardly directed electrochemical proton gradient. Functional analysis in *Xenopus* oocytes and in mammalian cells established that PepT1 represents a low-affinity/high-capacity, and stereoselective transporter, with peptides that contain L-enantiomers of amino acid residues having a higher affinity for binding and transport than peptides that contain one or more D-enantiomers (Rubio-Aliaga and Daniel, 2002). A unique feature of PepT1 is the capability of sequence-independent transport of nearly all possible di- and tripeptides, including differently charged species (Daniel et al., 2006; Vig et al., 2006)

In addition to the large number of di- and tripeptides, peptidomimetics such as β-lactam antibiotics, selected angiotensin-converting enzyme inhibitors, some antiviral nucleoside prodrugs, and ω-amino fatty acids as well all serve as substrates for PepT1 (Rubio-Aliaga and Daniel, 2002).

In the course of the past few years, the study of intestinal peptide transport has rapidly evolved into a field of exciting nutritional and

biomedical applications. In particular, the effect of dietary protein on PepT1 expression and activity has been an area of active research in recent years. Results from these studies (for a review, see Daniel, 2004; Gilbert et al., 2008a) demonstrated that the expression level of PepT1, and its function is very responsive to dietary treatments. The corresponding information for teleost fish is completely unknown; however, this would be of great importance in particular for the farmed species raised in feed-based aquaculture systems. From the commercial perspective, it is very costly to use fish dietary protein as a nutrient, and even fractional improvements in this area have the potential to save the aquafeed industry millions of euros and also to reduce the amount of nitrogen excreted into the environment.

Among the teleosts, PepT1 has only been cloned and characterized in three species: zebrafish, *D. rerio* (Verri et al., 2003); Atlantic cod, *Gadus morhua* (Rønnestad et al., 2007b); and Asian weatherloach, *Misgurnus anguillicaudatus* (Gonçalves et al., 2007). In mammals, PepT1 is prominently expressed throughout the epithelium of the small intestine, where it is responsible for the transport of a significant fraction of dietary short-chain amino acids across the brush border membrane. Similarly, in fish, expression analysis at the mRNA level indicated that PepT1 is mainly expressed at the intestinal level, although moderate levels have also been detected in a variety of other tissues (Verri et al., 2003; Gonçalves et al., 2007; Rønnestad et al., 2007b). In marine fish larvae, PepT1 mRNA was expressed throughout the digestive system, except in the esophagus and sphincter regions (Amberg et al., 2008).

Accumulating evidence indicates that PepT1 may have a major role in fish nutrition (Dabrowski et al., 2005). It transports large amounts of amino acids in peptide form, thereby providing essential nutrients for fish growth and the required energy for metabolism and reproduction. However, to our knowledge, there is little information concerning the response of such transporter at the mRNA level to different feeding regimes in fish.

Molecular Cloning and Sequencing

At the beginning of our research, the coding sequences of two target genes, PepT1 and Δ6 desaturase, were not available in public databases for *D. labrax*. Considering this lack of information, a BlastN search was performed on the complete, nonredundant Genbank nucleotide database for orthologues of PepT1, and Δ6 desaturase in other fish and vertebrate species. A multiple sequence nucleotide alignment was then carried out on the coding sequences found for each gene, and a strategy based on regions of strong nucleotide conservation was used to design the primers. In the case of PepT1, for example, primer design was based on the alignment of three teleost PepT1 coding sequences available on the NCBI GenBank database: *G. morhua*, (accession no. AY621934), *Sebastes nebulosus* (accession no. EU160494), and *D. rerio* (accession no. AY300011). These presented several conserved regions within the sequence where primers could be reasonably designed (Terova et al., 2009).

Total RNA was extracted from sea bass tissues, reverse transcribed into cDNA, and amplified via PCR. The PCR products from PepT1, and Δ6 desaturase primer amplifications were then cloned and subsequently sequenced. Several cDNA fragments were obtained following this cloning strategy. Then, by assembly of the sequences of the partially overlapping clones, partial coding sequences of each gene were obtained. The full-length cDNAs of each gene were subsequently isolated by 5′- and 3′-RACE and deposited in GenBank under the accession no. FJ237043 for PepT1, and EU647692 for Δ6 desaturase (http://blast.ncbi.nlm.nih.gov).

In Silico Analysis

Oligopeptide Transporter PepT1

The amino acid sequence of sea bass PepT1 was analyzed using the open reading frame (ORF) finder program, which is available at NCBI

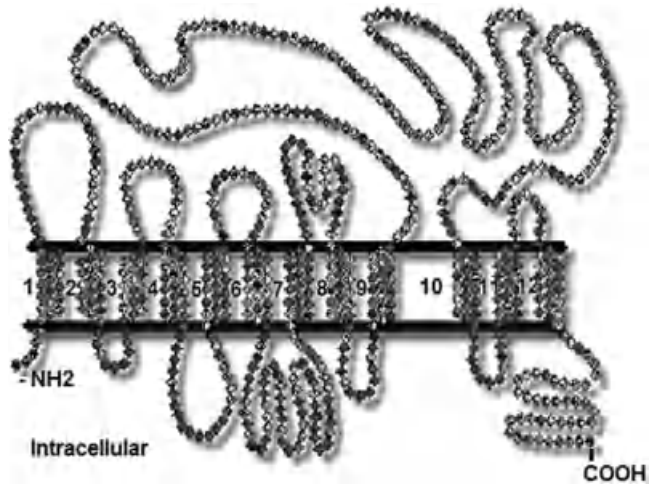

Figure 5.3 Membrane spanning helices in sea bass PepT1 protein predicted with the THMM program (http://www.cbs.dtu.dk/services/). See color insert.

(http://www.ncbi.nlm.nih.gov/projects/gorf/). Nucleotide sequences were compared with other sequences available at the GenBank database using the BLAST algorithm. Sequences were aligned using ClustalW program (www.ebi.ac.uk/clustalw) and Multiple Sequence Alignments Editor & Shading Utility, GeneDoc, version 2.6.002 (www.psc.edu/biomed/genedoc). Potential *N*-glycosylation, cAMP/cGMP-dependent protein kinase, and protein kinase C recognition sequences were identified using the PROSITE 20.40 computational tools (http://www.expasy.org/prosite/).

The transmembrane domains of sea bass PepT1 protein (accession no. FJ237043), predicted with THMM program (http://www.cbs.dtu.dk/services/), are presented in Figure 5.3. Sea bass PepT1 adopt a 12 transmembrane domain structure, with intracellular amino- and carboxyl-terminus, a large extracellular loop between the membrane spanning helices (MSH) 9 and 10, and an elongated intracellular loop between MSH 6 and 7. Three putative extracellular *N*-glycosylation sites and four putative intracellular cAMP/cGMP-dependent protein kinase A were identified (Terova et al., 2009).

The predicted sea bass PepT1 amino acid sequence shows extensive sequence similarity to human and other vertebrate PepT1 in the first four amino-terminal transmembrane regions as well as in the regions from the seventh to ninth domain, whereas there are variations in the large extracellular loop between transmembrane domains 9 and 10. In fact, the length of this loop is 200 amino acid (aa) residues in sea bass, 187 aa in cod, 240 aa in China rockfish, 148 aa in zebrafish, 242 aa in frog, 205 aa in dog, 204 aa in chicken, 203 aa in sheep and cattle, 236 in turkey, 239 in rabbit, 204 in monkey, human, and pig, and 240 aa in rat and mouse.

To address the structure–function properties of mammalian peptide transporters, Doring et al. (1996), Fei et al. (1997), and Terada et al. (2000) generated a range of chimeric transporters in which regions of mammalian PepT1 were replaced with the corresponding regions from PepT2 and vice versa. The distinctly different affinities for the same substrates of PepT1 and PepT2 were used to define the obtained phenotype and to draw conclusions about important protein regions. The functional characteristics of these chimeras were studied after expression in *Xenopus* oocytes. These experiments suggested that

the first four amino-terminal transmembrane regions as well as MSH 7–9 are important regions in which substrate binding occurs and in which most PepT1 functions are encoded. The role of the large extracellular loop between transmembrane domains 9 and 10 is unknown, and only one highly conserved residue (E595, numbering according to human PepT1) in this C-terminal half of PepT1 has proven to be of functional relevance (Bolger et al., 1998).

The PepT1 amino acid sequence identity among the species was calculated using the ORF. Sea bass PepT1 showed the highest sequence homology with teleost (Atlantic cod, 68%; zebrafish, 65%; and China rockfish, 64%) and avian species (chicken, 61%; and turkey, 63%), and lower homology with amphibian and mammalian species (frog, 48%; dog, 59%; rabbit and rat, 57%; mouse, pig, cattle, sheep, rhesus monkey, and human, 58%).

Δ *6 Desaturase*

The full-length sea bass Δ6 desaturase cDNA (Accession no. EU647692) consists of 2162 bp, comprising a 5′-untranslated region of 351 bp, an ORF of 1338 bp, and a 3′-untranslated region of 472 bp, including the possible polyadenylation signal (AATAAA). The deduced amino acid sequence shows that sea bass Δ6 desaturase is 445 amino acid long protein with a predicted molecular mass of approximately 51.7 kDa.

The amino acid sequence identity among the species was calculated using the ORF. Sea bass Δ6 desaturase protein showed a high percentage of identity both with other teleost Δ6 desaturases (70–90%) and with orthologous of the same protein in higher vertebrates (60–70%).

Phylogenetic Analysis

To analyze the evolutionary relationship of sea bass PepT1 with respect to other publicly available, related genes in other teleosts and vertebrate species, we reconstructed a phylogenetic tree (Figure 5.4). These analyses were computed by the neighbor-joining method (Saitou and Nei, 1987), as implemented in MEGA 4.0 (Tamura et al., 2007).

The clustering pattern provides evidence that sea bass PepT1 (Figure 5.4) is grouped with high bootstrap support in the lineage of other teleosts, sharing the highest homology with Atlantic cod PepT1, whereas the avians (chicken, turkey) and the mammalians (human, rhesus monkey, dog, rat, mouse, cattle, pig, sheep) are grouped into two other distinct lineages.

Dietary Manipulation of PepT1 and Δ 6 Desaturase Gene Expression

After the identification of the cDNA sequences of PepT1 and Δ6 desaturase by molecular cloning and sequencing, we assessed by real-time RT-PCR, the impact of chronic feed deprivation and successive refeeding, on mRNA copy number of both genes, in sea bass. The aim of this quantification was to relate these transcript levels to the feeding status of the animals. For this purpose, a compensatory growth experiment was conducted. Briefly, one month prior to starting the experiment, 140 sea bass were randomly stocked into four tanks of 2 m^3 each, with 35 fish per tank and allowed to acclimate. During the one-month acclimation period, all fish were fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system. Other water conditions were as follows: temperature, $20 \pm 2^{\circ}C$, pH 7, and total ammonia < 0.2 mg/L; dissolved oxygen was maintained over 99% of the saturation by insufflating pure O_2 to the system.

At the start of the experiment, all the fish were weighed, and two of the tanks were randomly assigned to each of two treatments. Fish in these two tanks were fed to apparent satiety (fed control), whereas fish in the other two tanks were deprived of food for 35 days and then refed to apparent satiety for 21 days with the same type of feed utilized before fasting.

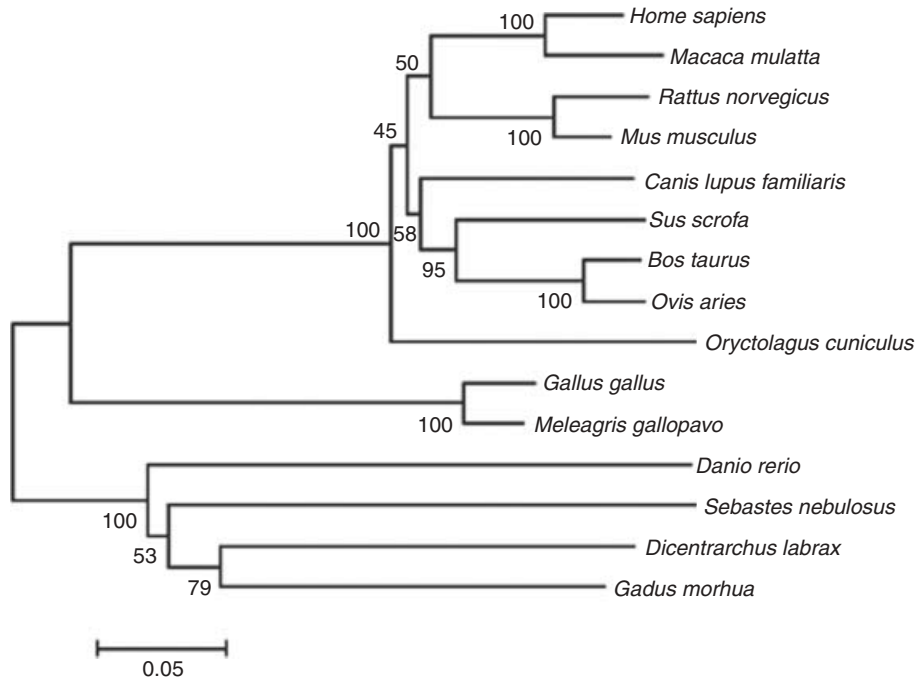

Figure 5.4 Unrooted phylogenetic tree depicting the evolutionary relationship of vertebrate PepT1 transporters. The unrooted tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on the alignment of the complete amino acid sequences of known vertebrate PepT1 transporters. Bootstrap values (1000 replicates) indicating the occurrence of nodes are reported above each branch in the figure (modified from Terova et al., 2009). GenBank accession numbers for amino acid sequence comparisons are: AAY17354 [Atlantic cod PepT1 (Rønnestad et al., 2007a)], ABV82968 (China rockfish Pept1), AAQ65244 [zebrafish PEPT1 (Verri et al., 2003)], AAK39954 [chicken PepT1 (Chen et al., 2002)], AAO16604 (turkey PepT1), AAA17721 [rabbit PEPT1 (Fei et al., 1994)], NP_001003036 (dog PEPT1), AAO43094 [pig PEPT1 (Klang et al., 2005)], XP_599441 (cattle PEPT1), AAA63797 [human PEPT1 (Liang et al., 1995)], NP_001028071 [macaque PEPT1 (Zhang et al., 2004)], BAA09318 [rat PEPT1 (Miyamoto et al., 1996)], NP_444309 [mouse PEPT1 (Fei et al., 2000)], AAK14788 [sheep PEPT1 (Mathews et al., 1996; Pan et al., 2001)].

Feed consumption (g) in each tank was estimated from the difference between feed delivered into the tank and uneaten feed that was collected from the bottom of the tank. Feed intake was converted to grams of feed consumed per kg body weight (BW) of the fish per day. Five fish from each of the experimental groups were sampled at the following time points: before fasting (day 0), 4 days after fasting, at the end of fasting, and then sequentially at 4, 14, and 21 days following refeeding. Fish were sampled 15 min before the scheduled feeding time. For the molecular biology analysis, several tissues were isolated and stored at -80°C.

All the fish in the tanks were also weighed at the end of fasting period and after 21 days of refeeding. They were rapidly anesthetized with tricainemethane sulfonate and BW was measured.

Effect of Dietary Manipulation on Fish Growth Performances

At the beginning of the experiment, the mean BW of the sea bass was 120.11 ± 4.47 g for the control group and 117.58 ± 3.58 g for the "food deprived" group (Terova et al., 2009). After

35 days of fasting, the mean BW and condition factor of fasted fish was significantly lower ($p < 0.01$) than that of the fed controls. During the subsequent refeeding period, previously unfed fish increased the BW sufficiently to overcome the weight loss caused by the 5-wk feed restriction. Fish that had experienced feed deprivation exhibited higher feeding rates than ad libitum fed controls during the first 2 wk of refeeding ($p < 0.05$). Refeeding of sea bass after 35 days of starvation was marked by hyperphagia as early as the first day. However, the hyperphagic period was shorter than the fasting period.

Effect of Dietary Manipulation on Gene Expression

Total RNA extracted from sea bass tissues was subjected to real-time RT-PCR using the absolute mRNA quantification method. The absolute number of each gene transcript copies was quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of that gene. For example, in the case of PepT1, a forward and a reverse primer were designed based on the mRNA sequences of the *D. labrax* PepT1 we had identified (accession no. FJ237043), flanking a region of 400 bp of the PepT1 gene. This primer pair was used to create templates for the in vitro transcription of mRNAs for PepT1: The forward primer was engineered to contain a T7 phage polymerase promoter gene sequence to its 5′ end and used together with a reverse primer in a conventional RT-PCR of total RNA extracted from sea bass proximal intestine RNA. RT-PCR products were then evaluated on agarose gel stained with ethidium bromide, cloned, and subsequently sequenced. In vitro transcription was then performed using T7 RNA polymerase.

The molecular weight (MW) of the in vitro-transcribed RNA was calculated according to the following standard formula where PepT1 is reported as an example: PepT1 MW = [129 (number of A bases × 329.2) + 69 (number of U bases × 306.2) + 66 (number of C bases × 305.2) + 98 (number of G bases × 345.2)] + 159. The result was 157705.2. Spectrophotometry at 260 nm gave a concentration of 528 ng/μL for PepT1. Therefore, the concentration of the final working solution was 2.02E + 12 molecules/μL. The same procedure was applied for the in vitro transcription of mRNAs of the other target gene: Δ6 desaturase.

The mRNAs produced by in vitro transcription were then used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs at tenfold dilutions were subjected in triplicate to real-time PCR using one tube-two step TaqMan RT-PCR. The Ct (cycle threshold) values obtained were then used to create the standard curve.

For the quantitation of each gene transcripts, an aliquot of total RNA extracted from the experimental samples was subjected, in parallel to standard mRNAs of that gene, to real-time PCR under the same experimental conditions as used to establish the standard curves. Real-time Assays-by-Design℠ PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI). TaqMan® PCR was performed on an ABI Thermocycler too. Data from the Taqman® PCR runs were collected with ABI's Sequence Detector Program. The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

Δ 6 Desaturase

The absolute mRNA levels of Δ6 desaturase in liver and proximal intestine in response to the feeding trial are presented in Figures 5.5 and 5.6, respectively. In both tissues, the analysis showed significant changes in Δ6 desaturase expression levels during the experiment. In sea bass liver, fasting for 35 days significantly increased the amount of Δ6 desaturase transcripts ($p < 0.05$). The subsequent recovery from fasting was associated with a significant

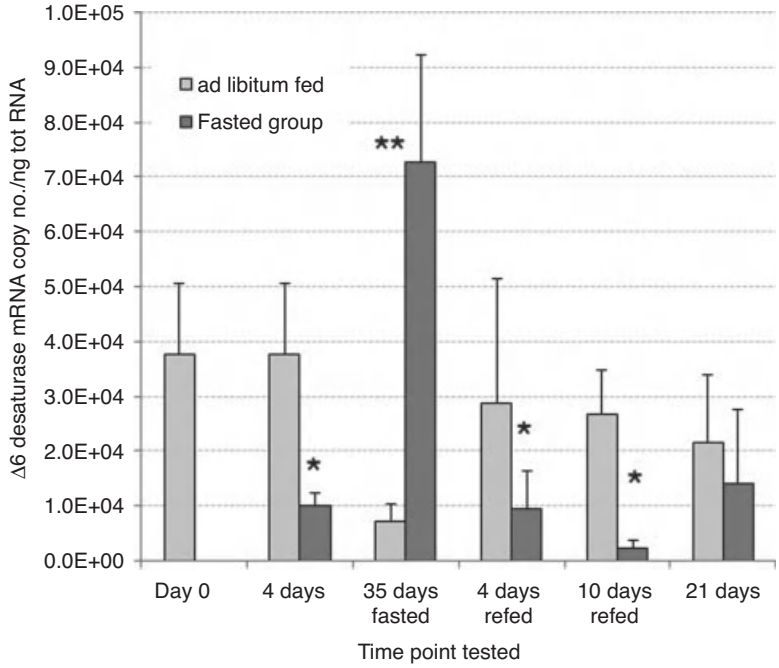

Figure 5.5 Expression levels of Δ6 desaturase measured by real-time PCR in sea bass liver. Δ6 desaturase mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, and 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Asterisk (*) indicates significantly different means from controls for each time point tested ($p < 0.05$).

decrease in Δ6 desaturase mRNA transcript levels, compared to control fish, until the tenth day of refeeding. At the end of the refeeding period, the expression of Δ6 desaturase increased to levels comparable to control (Figure 5.5).

Conversely, prolonged fasting caused a significant decrease in Δ6 desaturase transcripts in the proximal intestine as early as 4 days of fasting ($p < 0.05$) (Figure 5.6). The recovery from fasting was associated with a significant increase in mRNA transcript levels, beyond control levels, until the end of the fourth day of refeeding, in support of compensatory growth. Subsequently, 10 days after refeeding, the Δ6 desaturase mRNA copy number levels decrease compared to the previous time point tested, and then returned similar to controls at the end of 21 days of refeeding.

PepT1

Real-time RT-PCR quantification of PepT1 mRNAs revealed the following spatial distribution of PepT1 gene expression in sea bass digestive tract (Figure 5.7): high levels of expression in segments 1–3 (the first 3 cm) of the proximal intestine, lower levels in pyloric caeca and intestinal segments 4–10, and very low levels in gastroesophageal junction, stomach fundus, pyloric antrum, most distal intestinal region (segments 9–10), and rectum (Terova et al., 2009).

Thus, it is likely that, in sea bass, the proximal intestine is the main expression and production site of PepT1, as also demonstrated in humans (Ford et al., 2003) and other fish species such as cod (Rønnestad et al., 2007a), weatherloach (Gonçalves et al., 2007), and zebrafish (Verri

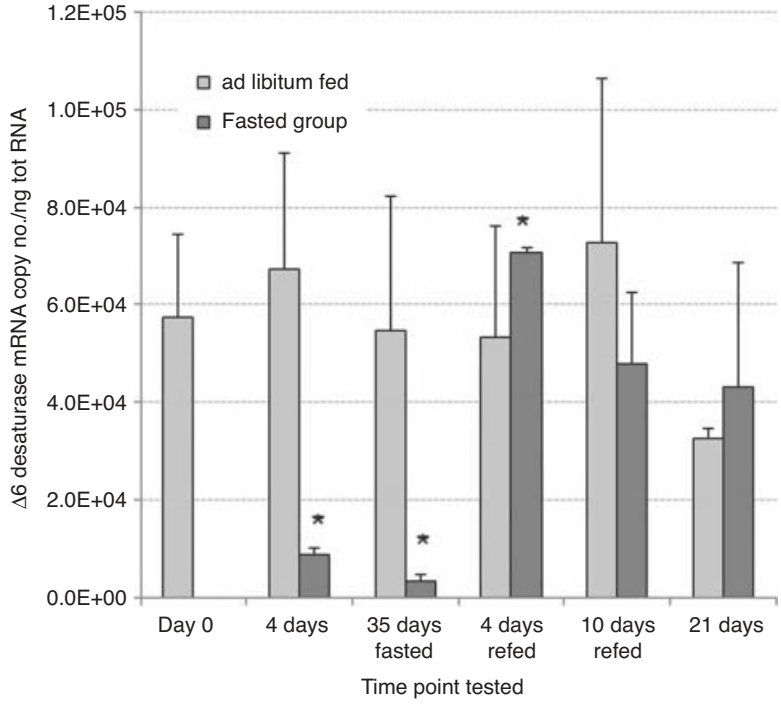

Figure 5.6 Expression levels of Δ6 desaturase measured by real-time PCR in sea bass proximal intestine. Δ6 desaturase mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, and 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Asterisk (*) indicates significantly different means from controls for each time point tested ($p < 0.05$).

et al., 2003). The presence of PepT1 mRNA in pyloric caeca and in the subsequent 10-cm region of the intestinal tissue, at albeit differing levels, is similar to the spatial distribution of PepT1 mRNAs found in Atlantic cod intestine (Rønnestad et al., 2007a). In addition, the relatively very low level of expression that we found in sea bass gills, heart, liver, and stomach matches the pattern reported for Atlantic cod (Rønnestad et al., 2007a). Sites with lower levels of PepT1 expression or activity, or both, are also found in human colon and bile duct epithelium and in rat brain and liver (Miyamoto et al., 1996; Knutter et al., 2002; Ford et al., 2003).

To study the dietary regulation of PepT1 mRNA levels in *D. labrax,* the fish were starved for 35 days and then refed for 3 weeks. PepT1 mRNA levels in the proximal portion of the intestine had not changed after 4 days of fasting, but were downregulated after 5 weeks of fasting (Terova et al., 2009). These results do not match those of previous studies that investigated the behavior of the transcripts of this gene over brief fasting periods in mammals or in birds. Short-term starvation in rats has been shown to increase mRNA and protein expression of PepT1: in the study of Ihara et al. (2000), mRNA and protein expression were increased by 179% in rats starved for 4 days, whereas in the study of Thamotharan et al. (1999), PepT1 mRNA and protein increased threefold, and the rate of peptide transport increased dramatically after only 1 day of fasting. Similarly, Gilbert et al. (2008b) found that feed restriction

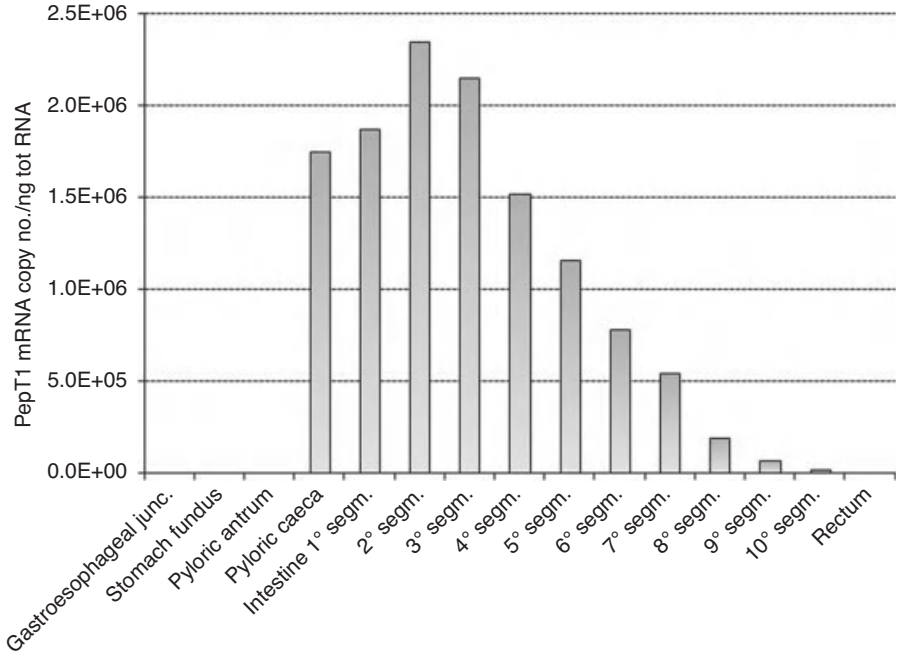

Figure 5.7 Spatial distribution of sea bass PepT1 mRNA in the digestive tract as determined by real-time quantitative PCR. Expression levels of PepT1 measured by real-time PCR in the different consecutive segments of the sea bass digestive tract. PepT1 mRNA copy number was normalized as a ratio to 100 ng total RNA. (Modified from Terova et al., 2009.)

increased expression of PepT1 mRNA in broilers aged 3–14 days after hatching.

The different fasting-induced changes in PepT1 mRNA expression in sea bass may reflect the impact of a more substantial fasting period in fish as compared to mammals and birds. Maybe the duration of fasting in the studies conducted in rats and birds was not long enough to generate the magnitude of nutritional insult necessary to downregulate PepT1 mRNA. However, future studies are needed to answer the question of whether the increases in PepT1 expressions observed in rats and birds would have persisted if fasting had been prolonged.

On the other hand, fasting in our study was associated with significant changes in two of the growth indexes examined: BW and condition factor (an indicator of body shape), which suggests that animals were duly stressed (Terova et al., 2009).

However, we cannot extensively discuss the potential role of the decrease in PepT1 mRNA levels based on the results of the present study, as we do not know whether the mRNA profile is consistent with the functional protein levels. Therefore, without protein data, we cannot draw any conclusions about the relationship between the mRNA levels and the transport activity in the intestine of fasted sea bass. Thus, our aforementioned hypothesis together with the notion that the different fasting-induced changes in PepT1 mRNA expression may also reflect differences in energy metabolism between homeotherms (mammals and birds) and poikilotherms (fish) must be confirmed by further investigations.

Short-term refeeding (4 days) promoted a remarkable increase in the transcription levels of PepT1 mRNA, which significantly exceeded the levels of the controls. This result might be

explained by the great quantity of protein taken in with the food in the initial days of refeeding. In fact, refeeding of sea bass after a long starvation period was marked by hyperphagia as early as the first day.

A long fasting period, like that in our study, appears to require rather long times for the PepT1 transcripts to return to "normal." In fact, the levels of mRNA only reached the control mRNA levels at the end of the third week of refeeding. Perhaps, the extent to which fasted fish display high levels of PepT1 expression upon return to adequate feeding conditions is related to the severity of the nutritional insult sustained by the animals. However, additional studies are needed to confirm this hypothesis in fish as this is actually the first to have investigated PepT1 expression patterns for such a long fasting period.

Conclusions

In conclusion, we have isolated the complete coding sequences for Δ 6 desaturase and PepT1 in sea bass (*D. labrax*). The nucleic and amino acid sequences of both are highly similar to the previously identified teleost, avian, and mammalian respective orthologs. We have also demonstrated that the nutritional state of the animal influences the levels of expression of the aforementioned genes. Taken together, these data may indicate that these genes participate in promoting sea bass compensatory growth induced by refeeding. However, future studies are necessary to completely clarify the underlying mechanism of each gene activation in sea bass. Indeed, the present study is the first one to investigate the behavior of the transcripts of those genes over such a long period of fasting and subsequent refeeding.

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Chapter 6

Functional Genomic Analysis of the Nutritional and Hormonal Regulation of Fish Glucose and Lipid Metabolism

Sandrine Skiba-Cassy, Sergio Polakof, Iban Seilez, and Stéphane Panserat

Abstract: It is now recognized that the replacement of fishmeal and fish oil with plant raw materials represents a sustainable alternative for the stability and further expansion of aquaculture. So, in this context, exploring the consequences of such dietary changes on the regulation of fish metabolism bears both scientific and practical relevance. The regulation of metabolism is achieved by coordinated actions between tissues and molecular mechanisms occurring at the cellular level. Enzymes that catalyze metabolic reactions are regulated at several levels, including both the amount of enzymes and the activities of the enzymes. The regulation of expression of metabolic enzymes involved complex interactions of hormonal and nutritional factors. Although not as widely recognized as hormones (insulin, IGFs, glucagon, somatostatins), nutritional signals play an important role in the control of metabolism. In the last several years, significant progress has been made in understanding the molecular mechanisms involved in the control of mammalian metabolism-related gene expression in response to major macronutrients (carbohydrates, fatty acids, and amino acids) in interaction with hormones, especially insulin. In this chapter, we review how fish metabolism is regulated at gene expression level by nutritional and hormonal factors and what are the main intracellular signaling pathways involved in these regulations. This review will cover genes related to carbohydrate (glycolysis and gluconeogenesis), lipid (fatty acids biosynthesis, lipogenesis, lipolysis, and fatty acid oxidation), and protein metabolism.

Introduction

Most teleost fish that are reared at commercial scale are adapted to using amino acids as preferred energy source over carbohydrates and thus require a dietary supply of high levels of amino acids. In intensive feed-based aquaculture, this requirement is met with diets predominantly based on fishmeal, but the sustainability of this practice that requires high quantities of wild fish for feed is now reappraised since such marine resources are not extendable. It is now recognized that the replacement of fishmeal with plant raw materials represents a sustainable alternative for the stability and further expansion of aquaculture. However, such substitution remains problematic since feedstuffs of plant origin have different amino acid

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

profiles compared with fishmeal and are naturally rich in carbohydrates. The presence of high levels of carbohydrates is highly problematic since carnivorous fish are recognized for their low efficiency in using digestible carbohydrates and are often described as "glucose intolerant" (Moon, 2001). So, in this context, exploring the consequences of such dietary changes on the regulation of fish metabolism bears both scientific and practical relevance.

Metabolism is defined as the sum of chemical reactions allowing the conversion of a particular molecule into some other molecule and happening in living organisms to maintain life. These reactions are classically organized in several pathways that can be divided into two broad classes: those that convert energy into biologically useful forms and those that require inputs of energy to proceed. The reactions that transform fuels into cellular energy are called catabolic reactions. On the other hand, reactions that require energy such as the synthesis of carbohydrates, fat, and protein are called anabolic reactions. The regulation of metabolism is achieved by coordinated actions between tissues and molecular mechanisms occurring at the cellular level. Enzymes that catalyze metabolic reactions are regulated at several levels, including enzymatic activities and the amount of enzymes. The regulation of expression of metabolic enzymes involves complex cross talking between hormonal and nutritional factors. Although not as widely recognized as hormones (insulin, IGFs, glucagon, somatostatins, etc.), nutritional signals play an important role in the control of metabolism. In the last several years, significant progress has been achieved in understanding the molecular mechanisms involved in the control of fish metabolism-related gene expression in response to macronutrients, including carbohydrates, fatty acids, and amino acids. In the present chapter, we review how fish metabolism is regulated by nutritional and hormonal factors at the gene expression level and what are the main intracellular signaling pathways involved in these regulations. This review will enclose genes related to carbohydrate (glycolysis and gluconeogenesis) and lipid (fatty acids biosynthesis and lipogenesis) metabolisms.

Carbohydrate Metabolism

Glycolysis

Glycolysis represents the only metabolic pathway leading to glucose catabolism in all organisms including fish (Cowey and Walton, 1989). It consists of a sequence of reactions occurring in the cytosol, which convert one molecule of glucose (6C) into two molecules of pyruvate (3C) with the concomitant generation of two molecules each of ATP and NADH. All enzymes involved in the glycolysis have been reported in fish (Walton and Cowey, 1979) although only those catalyzing irreversible reactions are involved on its regulation, including hexokinases (HK), phosphofructose-1-kinase (6PF1K), and pyruvate kinase (PK; Figure 6.1).

HK catalyze the first reaction of glycolysis by phosphorylating glucose into glucose-6-phosphate (G6P), an intermediary molecule for other metabolic pathways such as glycogenesis and the pentose phosphate pathway. Four closely related HK (HK I–IV) have been described in mammals (Wilson, 1995; Cardenas et al., 1998) and differ by their tissue distribution and functional properties; thus, HK-IV or glucokinase (GK) is characterized by a low affinity for glucose (K_m 5–12 mM), no inhibition by G6P, and a molecular weight of 50 kDa rather than the 100 kDa of HK-I, which is ubiquitously distributed in vertebrate tissues and characterized by a relatively high affinity for glucose (low K_m) and inhibition by G6P and inorganic phosphate. In the muscle, HK-II is predominantly expressed, whereas HK-IV also known as GK is the main hexokinase present in the liver (Iynedjian, 2009).

GK has been characterized in several fish species, most of them paradoxically carnivorous, with a natural low intake of carbohydrates. GK has been characterized in

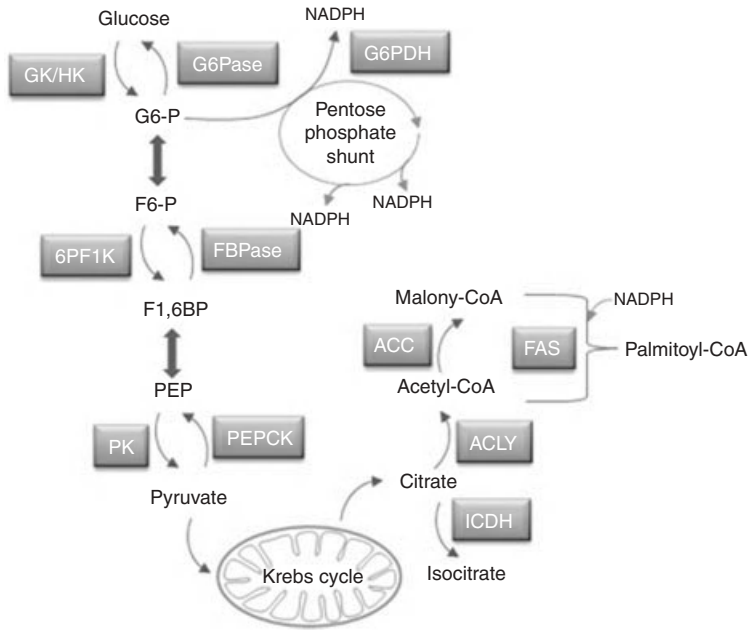

Figure 6.1 Schematic representation of metabolic pathways involved in glycolysis, gluconeogenesis, pentose phosphate shunt, and lipid synthesis. Glycolytic enzymes: glucokinase (GK), 6-phosphofructo-1-kinase (6PF1K), and pyruvate kinase (PK). Gluconeogenic enzymes: phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase). Pentose phosphate shunt enzymes: glucose-6-phosphate dehydrogenase (G6PDH). Lipogenic enzymes: ATP-citrate lyase (ACLY), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and isocitrate dehydrogenase (ICDH). G6-P, glucose-6-phosphate; F6-P, fructose-6-phosphate; F1,6BP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate. See color insert.

Atlantic salmon (*Salmo salar*; Tranulis et al., 1996), tilapia (Joy, 2002), Atlantic halibut (*Hippoglossus hipoglossus*; Tranulis et al., 1997), whitefish (*Erythroculter ilishaeformis*; Ge et al., 2006), grass carp (*Ctenopharyngodon idella*; Gao et al., 2010), goldfish (*Carassius auratus*; Polakof et al., 2009a), zebrafish (Gonzalez-Alvarez et al., 2009), perch (Borrebaek and Christophersen, 2000), rainbow trout, gilthead sea bream, and carp (Blin et al., 1999). Particular attention has been paid to the regulation of GK gene expression, and as previously reported in mammals, GK gene expression is also altered by feeding conditions in fish. In rainbow trout, food deprivation leads to decreased expression of GK, whereas refeeding increased it (Soengas et al., 2006; Skiba-Cassy et al., 2009). Similarly, starvation and energy restriction decreased hepatic GK mRNA levels in gilthead sea bream (*Sparus aurata*; Caseras et al., 2000) and zebrafish (Gonzalez-Alvarez et al., 2009). These results suggest strong relationship of liver GK with the circulating glucose levels and with the glycogen control, as occurs in mammals (Iynedjian, 2009). Several data obtained in different fish species tend toward the same conclusion that carbohydrates are the main regulator of GK gene expression in fish. Actually, GK gene expression is induced in rainbow trout, gilthead sea bream, and common carp by a carbohydrate-rich diet containing more than 20% digestible starch (Panserat et al., 2000, 2002b; Meton et al., 2004). GK mRNA expression also increased in rainbow trout according to the proportion of dietary starch content (Capilla et al., 2003), and it was

similarly reported that gilthead sea bream fed high-carbohydrate/low-protein diet presented high hepatic GK gene expression, while GK mRNA levels were hardly quantified in fish fed conversely low-carbohydrate/high-protein diet (Caseras et al., 2002; Meton et al., 2004). Direct evidence of the involvement of glucose in the positive regulation of GK gene expression comes from data indicating that a single meal containing 24% of glucose-induced hepatic GK gene expression in rainbow trout. Further confirmation for glucose role as GK regulator was recently found in rainbow trout (Polakof et al., 2009b) and zebrafish (Gonzalez-Alvarez et al., 2009) receiving intraperitoneal administration of glucose.

Insulin is a major regulator of GK gene expression in mammalian liver (Iynedjian et al., 1989). In fish, insulin is able to regulate GK gene expression, but its action needs to be clarified. As in mammals (see above), insulin administration stimulates GK expression in gilthead sea bream liver (Egea et al., 2007). In primary cell culture of rainbow trout hepatocytes, insulin is able to stimulate GK gene expression, but the presence of amino acids seems to be necessary; otherwise, GK gene expression is repressed by insulin (Plagnes-Juan et al., 2008; Lansard et al., 2010b). The inhibitory effect of insulin on GK gene expression was further confirmed in vivo following acute intraperitoneal administration of insulin or chronic infusion of insulin (4 days) in both fasted and high-carbohydrate fed fish (Polakof et al., 2010b, 2010d). The in vivo repression of GK gene by insulin in trout liver represents a paradoxical regulation in a fish species in which the natural carbohydrate intake is very low and the insulin secretion is well stimulated in fish fed high carbohydrate diets (Capilla et al., 2003).

The regulation of the other HKs in fish is less known when compared with the information available for GK. For instance, HK I has been described and sequenced in carp, rainbow trout, sea bream (Blin et al., 1999; Soengas et al., 2006) and cod (*Gadus morhua*; Hall et al., 2009). Although, in general, HK-I have been described as ubiquitous, Soengas et al (2006) have reported an HK-I-L (ubiquitous) and another HK-I-H (not expressed in liver or erythrocytes), also consistent with the HK-Ia and HK-Ib described by Hall et al. (2009) in cod tissues. HK-I and HK-II gene expression was also found in zebrafish tissues (Gonzalez-Alvarez et al., 2009). In opposition to the nutritional regulation of the GK, the other HKs seems to be constitutively expressed, being unaffected by the food deprivation in zebrafish (Gonzalez-Alvarez et al., 2009), Atlantic cod (Hall et al., 2009), and trout (Soengas et al., 2006).

Within the glycolytic pathway, 6-Phosphofructo-1-kinase catalyzes the phosphorylation of fructose-6-phospate into fructose-1, 6-bisphosphate. As in mammals, two isoforms of this enzyme have been found in fish tissues, including the 6PF1K-L (liver) and 6PF1K-M (muscle) (van der Meulen et al., 2006; Mediavilla et al., 2007; Polakof et al., 2009b). Since this enzyme is mainly regulated allosterically, most of the investigations conducted were focused on the activity level (for review, see Enes et al., 2009) and then very little data is available regarding the regulation at the molecular level. For instance, studies conducted to investigate the phosphorylation status of the protein indicate that in liver the dephosphorylated form is the more active (Su and Storey, 1994), while in muscle phosphorylation by cAMP-dependent protein kinase increases 6PF1K activity (Mediavilla et al., 2007). Conversely, expression of 6PF1K was significantly enhanced by intraperitoneal administration of glucose to rainbow trout (Polakof et al., 2009b), whereas acute or chronic administration of insulin failed to regulate expression of 6PF1K both in liver and muscle of fasted and high-carbohydrate fed fish (Polakof et al., 2010b, 2010d). The regulation of 6PF1K gene expression was also investigated in vitro using primary cell culture of hepatocytes; in this cellular model, expression of 6PF1K is positively regulated by the combination of insulin and amino acids (Lansard et al., 2010b).

The biochemical and molecular regulation of this enzyme by pharmacological doses of cortisol was conducted on *Fundulus heteroclitus* (Lawrence DeKoning et al., 2004). However, the unrelated changes in both activities and mRNA levels found in muscle made difficult to interpret this data, and further studies are needed, probably including physiological hormone levels. 6PF1K was also characterized at molecular level in the Baikal yellowfin (*Cottocomephorus grewingki*; Tulokhonov et al., 1996) and zebrafish (6PF1K-M; van der Meulen et al., 2006). In the later, unregulated gene expression was found in fish subjected to continuous exercise, suggesting a constitutively enhanced glycolytic pathway under such circumstances.

PK is the last enzyme of the glycolytic pathway that catalyzes the conversion of phosphoenolpyruvate to pyruvate. Mammalian PK is encoded by two genes named PKL and PKM, which produce the L- and R-type isoenzymes by means of alternative promoters, and the M_1- and M_2-types by mutually exclusive alternative splicing, respectively (Yamada and Noguchi, 1999). In mammals, PK activity and gene expression in the liver is mainly attributed to PK-L isoenzyme. PK gene expression decreases during starvation, whereas it increases following high carbohydrate feeding. PK-L and PK-M were characterized in rainbow trout (Polakof et al., 2009b) and globefish (*Fugu rubripes*; Ohta et al., 2003). In rainbow trout, PK gene expression is poorly affected by the nutritional state (Panserat et al., 2001b), except a significant upregulation in muscle after 30 days of starvation in trout (Johansen and Overturf, 2006). In this study, fish refed after the prolonged starvation recovered the PK mRNA basal levels. Moreover, the presence of high proportion of dietary carbohydrates or the substitution of fishmeal and fish oil by plant products in fish diets do not modify PK gene expression (Panserat et al., 2001b; Kirchner et al., 2003b; Lansard et al., 2009; Skiba-Cassy et al., 2009), whereas activity of PK is induced by dietary carbohydrates in several fish species (for review, see Enes et al., 2009). This suggests that PK activity is poorly regulated at molecular level in fish. In mammals, insulin and glucagon represent two hormonal factors involved in the regulation of hepatic PK gene expression and presented opposite effects: insulin treatment stimulates PK gene expression, whereas glucagon inhibits PK mRNA accumulation by decreasing gene transcription (Pilkis and Granner, 1992). In rainbow trout, acute administration of insulin failed to stimulate PK gene expression in liver (Plagnes-Juan et al., 2008; Polakof et al., 2010d), whereas it faintly decreased PK mRNA level in skeletal muscle. When insulin is chronically administered to fasted and high-carbohydrate rainbow trout using osmotic pumps, PK gene expression becomes slightly reduced in liver but no more different from saline-treated fish in muscle (Polakof et al., 2010b, 2010d). All these data indicate that in vivo, exogenous insulin poorly affect PK gene expression. However, insulin is able to enhance PK mRNA accumulation in primary cell culture of hepatocytes, and this positive effect is enhanced by high concentration of glucose (Plagnes-Juan et al., 2008) or the presence of amino acids in the culture medium (Lansard et al., 2010b).

As indicated by in vitro studies, GK, 6PF1K, and PK gene expression were stimulated by the combination of insulin and amino acids compared with amino acids-alone stimulated cells. These data enlarge the roles of amino acids in fish from protein synthesis substrates and fuel sources to the regulation of metabolism-related gene expression. This action has probably to be linked to the signaling effect of amino acids well known in mammals (Dickinson and Rasmussen, 2011) and now also demonstrated in fish (Lansard et al., 2009). In fact, such upregulations were shown to implicate the activation of the TOR pathway, which also needs the combination of insulin and amino acids to be fully activated in fish as in mammals (Foster et al., 2010), since rapamycine totally abolished this positive effect (Lansard et al., 2010b).

Gluconeogenesis

Gluconeogenesis represents the endogenous production of glucose from nonglycosidic substrates including lactate, glycerol, and α-ketoacids. The rate of gluconeogenesis is controlled principally by the activities of unidirectional enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase), three enzymes that have been reported to exist in fish (Cowey and Walton, 1989; Figure 6.1).

PEPCK catalyzes the first reaction of glucoeneogenesis by converting oxaloacetate to phosphoenolpyruvate. PEPCK is present in two isoformes, one cytosolic and the other mitochondrial, encoded by two separate genes (Hanson and Reshef, 1997). The proportion of these isoforms in the liver depend on the species, being 100% cytosolic in rat (Ballard and Hanson, 1969; Cornell et al., 1986), 50%/50% in human (Brech et al., 1970; Diesterhaft et al., 1971), and 100% mitochondrial in avian species (Nordlie and Lardy, 1963; Elliott and Pogson, 1977). A full length PEPCK cDNA coding for a mitochondrial isoform has been cloned in rainbow trout liver (Panserat et al., 2001a). Partial sequences have also been characterized from common carp, gilthead sea bream (Panserat et al., 2002b), and zebrafish (Elo et al., 2007), but the nature of the isoform remains to be determined since the 5′ regions of the cDNAs allowing the differentiation of the isoform were not characterized.

PEPCK is not subjected to allosteric regulation or post-transcriptional regulation, and the major control of its activity take place by transcriptional regulation of PEPCK gene expression. In mammals, gene encoding the cytosolic isoform is under nutritional and hormonal control, which is not the case of the mitochondrial isoform, known to be constitutively expressed. In rainbow trout liver, PEPCK has been shown to be constitutively expressed independently of the nutritional status of the animal: unfed versus fed with or without carbohydrates or fed with increased dietary proportions of protein levels (Panserat et al., 2001a; Kirchner et al., 2003b; Plagnes-Juan et al., 2008; Lansard et al., 2009; Skiba-Cassy et al., 2009). This lack of nutritional regulation of PEPCK gene expression confirms the mitochondrial location of the PEPCK isoform cloned in rainbow trout. The absence of nutritional regulation of PEPCK gene expression was also observed in gilthead sea bream, where similar levels of PEPCK mRNA were observed over the post-prandial period, whatever the composition of the diet (Panserat et al., 2002b). On the contrary, in the herbivorous common carp, PEPCK gene expression seems to be regulated after feeding with lower levels of PEPCK mRNA 6 hours after feeding compared with 24 hours (Panserat et al., 2002b), suggesting that post-prandial regulation of PEPCK gene expression may differ between carnivorous and herbivorous fish species.

Although diet composition seems to barely affect PEPCK gene expression, individual nutrients are nevertheless able to influence PEPCK mRNA levels in primary cell culture of hepatocytes with a positive and negative effect of a pool of essential and nonessential amino acids (Lansard et al., 2010b) and glucose (Plagnes-Juan et al., 2008), respectively. The action of leucine, methionine, and lysine has also been analyzed in hepatocytes cell cultures, revealing distinct effects of these amino acids. Leucine inhibited the expression of PEPCK, as observed with a pool of amino acids, whereas lysine exhibited positive action. Finally, methionine did not exert any effect on PEPCK gene expression (Lansard et al., 2011). However, supplementation of rainbow trout diet with alanine, asparagine, or glutamine did not modify the hepatic level of expression of PEPCK (Kirchner et al., 2003a), suggesting that individual amino acids may play a role on PEPCK gene expression, but their action can be modulated by other factors, including hormones and interaction with other nutrients.

Hormonal regulation of PEPCK gene expression has been intensively investigated in

mammals. In these species, insulin inhibits expression of PEPCK at the transcriptional level (Hall, 2000) through the activation of the protein kinase Akt (Barthel and Schmoll, 2003). In rainbow trout, several studies indicated that acute administration of pharmacological dose of insulin induced Akt phosphorylation and reduced PEPCK mRNA level (Plagnes-Juan et al., 2008), as did the long-term intraperitoneal infusion of physiological dose of insulin (Polakof et al., 2010d). The inhibitory action of insulin of PEPCK gene expression was also confirmed in vitro using primary cell culture of hepatocytes (Plagnes-Juan et al., 2008; Lansard et al., 2010b). Paradoxically, the combination of insulin and carbohydrates during 5 days resulted in upregulation of the PEPCK gene in rainbow trout (Polakof et al., 2010b).

FBPase catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. In mammals, FBPase gene expression is regulated through both transcriptional and post-transcriptional mechanisms (Okar and Lange, 1999). FBPase gene expression has been demonstrated in the livers of rainbow trout (Panserat et al., 2001b), common carp, and gilthead sea bream (Panserat et al., 2002b). In rainbow trout, expression of FBPase seems to be poorly regulated by feeding or refeeding (Panserat et al., 2001b; Kirchner et al., 2008; Lansard et al., 2009), whereas starvation significantly increased expression of FBPase in zebrafish (Drew et al., 2008).

The effect of diet composition on FBPase gene expression has also been studied. Although introduction of carbohydrate in the diet did not change FBPase gene expression in the livers of rainbow trout and common carp (Panserat et al., 2001b, 2002b), expression of FBPase was lowered 6 hours after feeding in the liver of gilthead sea bream fed a carbohydrate-rich diet compared with fish fed without carbohydrates (Panserat et al., 2002b). It seems that FBPase is rather controlled by proteins than carbohydrates. Actually, at similar levels of carbohydrate intake, decreasing protein intake resulted in reduction of FBPase gene expression (Kirchner et al., 2003b), suggesting that proteins may regulate FBPase gene expression. Nevertheless, supplementation of diets with alanine, asparagine, or glutamine did not modify FBPase expression (Kirchner et al., 2003a). Similarly, stimulation of primary cell culture of rainbow trout hepatocytes with a pool of amino acids did not change FBPase mRNA level (Lansard et al., 2010b).

The effect of insulin has also been investigated both in vivo and in vitro. In vivo, contradictory results were obtained depending on the way of administration and the quantity of insulin used. For instance, the intraperitoneal administration of a pharmacological dose of insulin decreased expression of FBPase (Plagnes-Juan et al., 2008), but similar acute treatment with physiological dose exhibited opposite effect (Polakof et al., 2010d). Inhibitory action of insulin can nevertheless be observed after long-term infusion of physiological quantity of insulin (Polakof et al., 2010d). In trout hepatocytes, insulin also failed to inhibit FBPase gene expression (Plagnes-Juan et al., 2008; Lansard et al., 2010b). A surprising positive effect of insulin is even observed when insulin is combined with a pool of amino acids (Lansard et al., 2010b). This regulation of FBPase gene expression by insulin and amino acids is nevertheless in agreement with the positive effect of dietary protein on hepatic FBPase gene expression. This gluconeogenesis potential promotion by the combination of insulin and amino acids also mimic the post-prandial state, which is characterized by hyperglycemia when carbohydrates are also included in the diet.

The production of free glucose from G6P, which is considered as the last step shared by both gluconeogenesis and glycogenolysis, is catalyzed by G6Pase. G6Pase constitutes an enzymatic complex that includes the glucose-6-phosphatase translocase that transports G6P from the cytoplasm to the lumen of the endoplasmic reticulum and the G6Pase phosphohydrolase (catalytic subunit) that resides in the lumen (Postic et al., 2004). The catalytic subunit of G6Pase has been partially cloned in rainbow

trout, common carp, and gilthead sea bream before being fully characterized from gilthead sea bream liver (Panserat et al., 2000, 2002b; Meton et al., 2004). This full-length cDNA encodes a 350 amino acids protein with low homology to mammalian G6Pase but correct conservation of key residues conferring to G6Pase its catalytic activity (Meton et al., 2004). According to ancestral whole genome duplication that has occurred in the teleost lineage following its divergence from mammals (Jaillon et al., 2004), multiple copies of genes may be found in fish. Analysis of the rainbow trout transcriptome databank recently reveals the existence of a second G6Pase cDNA with a different pattern of expression compared with the isoform initially cloned (Plagnes-Juan et al., 2008).

In mammals, the hepatic expression of G6Pase is subjected to hormonal and nutritional regulation. Starvation and hormones increasing cAMP stimulate G6Pase gene expression, whereas refeeding and insulin both develop opposite effects (Argaud et al., 1996; Minassian et al., 1999). Food deprivation resulted in increased hepatic expression of G6Pase in gilthead sea bream (Caseras et al., 2002; Kirchner et al., 2003a; Meton et al., 2004). In this species, one day is even sufficient to restore hepatic expression of G6Pase to levels measured in fed fish (Meton et al., 2004). In the same way, energy restriction was found to increase G6Pase mRNA levels in gilthead sea bream (Caseras et al., 2002). The results regarding the effect of starvation on the expression of G6Pase in rainbow trout liver are more controversial. Kirchner et al. (2005) demonstrated that even fish were fed or food deprived equivalent levels of G6Pase mRNA were measured in rainbow trout liver. Similarly, Panserat et al. (2000) indicated that feeding did not modify expression of G6Pase in rainbow trout liver when compared with fasted animals. On the contrary, expression of G6Pase was shown to decrease in refed rainbow trout compared with short-term fasted fish (Plagnes-Juan et al., 2008; Lansard et al., 2009; Skiba-Cassy et al., 2009). However, this decrease was only observed for one of the two isoforms of G6Pase present in this species, which may probably explain these discrepancies.

In mammals, G6Pase gene expression is downregulated by intake of carbohydrates through transcriptional mechanisms (Pilkis and Granner, 1992). The effect of dietary nutrients on G6Pase gene expression was also highly investigated in fish. Unlike mammals, a persistent high level of expression of G6Pase was recorded after a carbohydrate-rich meal in rainbow trout (Panserat et al., 2000; Kirchner et al., 2008) and common carp livers (Panserat et al., 2002b). The effect of dietary carbohydrates is much less clear in gilthead sea bream. Actually, Caseras et al. (2002) reported that G6Pase gene expression was not regulated by the amount of dietary carbohydrates in this species (Caseras et al., 2002) and Meton and coworkers indicated that refeeding after prolonged fasting rapidly decreased G6Pase gene expression irrespective of the proportions of proteins and carbohydrates introduced in the diet (Meton et al., 2004). On the contrary, Panserat et al. (2002b) indicated that expression of this gene was reduced 6 hours after feeding when fish were fed a diet containing 20% carbohydrates.

Dietary proteins have also been identified as regulators of G6Pase gene expression in fish. Decreasing dietary proteins intake while maintaining equivalent carbohydrate intake through pair feeding methods leads to reduced expression of G6Pase in rainbow trout liver (Kirchner et al., 2003b). The results are in agreement with the important positive effect of a mixture of amino acids on G6Pase gene expression observed in trout hepatocytes (Lansard et al., 2010b). However, contradictory results were obtained by Kirchner and collaborators in 2005 regarding the effect of dietary proteins on G6Pase gene expression (Kirchner et al., 2005). This discrepancy is probably caused by the different molecular methods used to evaluate G6Pase mRNA levels, Northern blot, and quantitative real time PCR, respectively. Northern blots have allowed quantification of mRNA encoding all isoforms of G6Pase,

whereas only one isoform has probably been detected by real-time PCR. The evidence that the two isoforms of rainbow trout G6Pase may be regulated in different ways also supports this speculation (Plagnes-Juan et al., 2008; Lansard et al., 2009; Skiba-Cassy et al., 2009). The effect of dietary carbohydrates has also been studied in gilthead sea bream. In this species, Enes and collaborators demonstrated that expression of G6Pase was not affected by the sources of carbohydrates (Enes et al., 2008).

Lipids may also interfere with G6Pase gene expression in rainbow trout. Therefore, fish fed high levels of lipids presented an inefficient control of their glycemia that seems to be related to increased expression of G6Pase mRNA (Panserat et al., 2002a).

In mammals, gluconeogenic enzymes are controlled at the transcriptional level by hormones, mainly insulin, glucagon, and glucocorticoids. Insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase, whereas glucagon and glucocorticoids stimulate hepatic glucose production by inducing these genes. Administration of a pharmacological dose of insulin led to reduced expression of G6Pase in rainbow trout and gilthead sea bream liver (Panserat et al., 2002a; Salgado et al., 2004; Plagnes-Juan et al., 2008), as did the long-term infusion of physiological dose of insulin (Polakof et al., 2010d). However, a surprising positive effect of insulin on G6Pase gene expression was also recorded after acute administration of physiological dose of insulin (Polakof et al., 2010d) in fasted fish, while the same dose injected to high-carbohydrate fed fish resulted in the oppose regulation (Polakof et al., 2010b). In vitro models of fish hepatocytes were also used to investigate the hormonal regulation of G6Pase gene expression. Therefore, as observed in mammalian models, insulin reduced G6Pase mRNA levels in rainbow trout and gilthead sea bream hepatocytes (Salgado et al., 2004; Plagnes-Juan et al., 2008; Lansard et al., 2010b), probably through the TOR signaling pathway at least in rainbow trout (Lansard et al., 2010b).

Lipid Metabolism

Biosynthesis of Long-Chain Polyunsaturated Fatty Acids

Stagnation in feed-grade fisheries, along with increased demand for fish oils, has dictated that alternative fish oils must be found if aquaculture is to continue to expand and supply more of the global demand for fish (Food And Agricultural Organisation of the United Nations (FAO), 2009; Naylor et al., 2009). The main sustainable alternative to fish oils is vegetable oils, which can be rich in C18 *n*-3 but devoid of the essential fatty acids such as eicosapentaenoic acid (EPA; C20 *n*-3) or docosahexaenoic acid (DHA; C22 *n*-3), compromising their nutritional value: EPA and DHA products are important nutrients for health (as main factors involved in protection against heart stroke, for example) and development (visual and cognitive development for example) in humans (Brouwer et al., 2006; Eilander et al., 2007). Understanding the molecular basis of EPA and DHA fatty acid biosynthesis and its nutritional regulation is indeed necessary to best exploit and eventually manipulate the activity of the fatty acid bioconversion pathways to enable efficient and effective use of vegetable oils in aquaculture.

The pathway whereby C20 and C22 fatty acids are biosynthesized from the C18 *n*-3 precursors is particularly important for fish (Tocher, 2010). Many vertebrates can convert the C18 *n*-3 fatty acid to long-chain *n*-3 fatty acids such as EPA and DHA via a pathway involving a series of fatty acid desaturation and elongation reactions in different tissues (mainly liver, intestine, and brain; Cook and McMaster, 2004; Tocher, 2010). Freshwater fish seem to express all the enzymes involved in long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis (different elongase and desaturase enzymes), whereas marine fish are unable to produce DHA at a significant rate because of apparent deficiencies in one or more steps in the pathway (Hastings et al., 2004; Morais et al.,

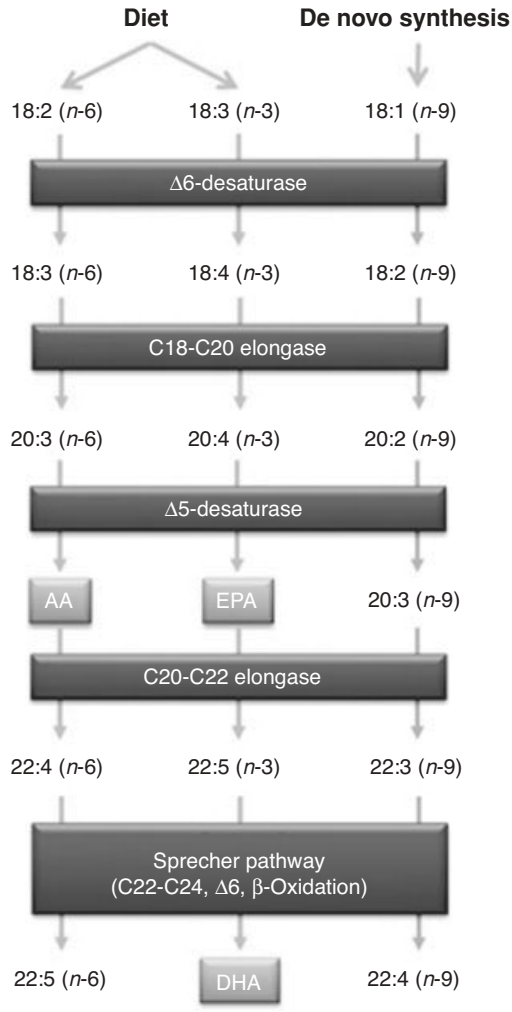

Figure 6.2 PUFA biosynthesis pathways. AA, arachidonic acid (20:4 *n*-6); EPA, eicosapentaenoic acid (20:5 *n*-3); DHA, docosahexaenoic acid (22:6 *n*-3). See color insert.

2009; Tocher, 2010). In recent years, significant progress has been made in characterizing at a molecular level fatty acid desaturase and elongase involved in LC-PUFA synthesis in fish species (Figure 6.2).

Cloned cDNAs for Δ 6 desaturase and elongases known to participate in HUFA biosynthesis were isolated and functionally tested in a number of different fish species, including freshwater species zebrafish, carp, and salmonids and marine species gilthead sea bream, cod, and turbot (Seiliez et al., 2001, 2003; Zheng et al., 2004a, 2004b; Morais et al., 2009). Interestingly, varying competencies of different fish species to biosynthesize PUFA may be related to the absence of some specific genes such as Δ 5-desaturase and elongases of very long-chain fatty acids ELOV1.2 in marine species as gilthead sea bream (Seiliez et al., 2003; Leaver et al., 2008). The expression of desaturase and elongase genes can be related to different diet composition and mainly to the dietary lipid profiles. Indeed, elongase and desaturase gene expression are higher in fish fed with diets poor in EPA and DHA sources (vegetable oils) and lower in fish fed fish oils rich in EPA and DHA (for review, see Tocher, 2010). Mechanisms of the regulation of these genes by nutrition are poorly studied in fish and presently cannot obtain high levels of EPA and DHA in flesh of fish fed alternative diets. Even though there are direct regulations by the nutrients (such as the class of lipids), our recent data have clearly shown that desaturase genes is positively regulated by insulin in liver of rainbow trout (Polakof et al., personal communication), which could be related to the well-known induction by feeding of gene expression. Better understanding of the regulation of the metabolic pathways is clearly needed.

Moreover, recent data about desaturase genes demonstrated the existence of paralogous genes coding for highly similar proteins in salmonids (Monroig et al., 2010). This could be due to the well-known recent duplication of the genome in the salmonid evolution (a post-tetraploidization genome). Screening of a genomic DNA library revealed the existence of at least four genes encoding putative desaturase proteins in Atlantic salmon. Tissue-specific expression is highly dependent on the desaturase genes as well as their nutritional regulation by dietary vegetable oils (Monroig et al., 2010), showing high levels of complexity for the nutritional regulation of the desaturase proteins. The observed differences in tissue expression

and nutritional regulation of many paralogous genes have to be taken into account when we analyze the gene expression in this fish species.

n-3 LC-PUFA are essential components of vertebrate membrane lipids and are now at critically low levels in modern Western diets. The main human dietary source for *n*-3 LC-PUFA is fish and seafood, and over 50% of global fish production is currently supplied by aquaculture. However, increasing pressure to include vegetable oils, which are devoid of *n*-3 LC-PUFA, in aquaculture feeds reduces their content in farmed fish flesh partially due to a low capacity of nutritional induction of LC-PUFA biosynthesis in fish. This is the reason why we will present here two alternative solutions to improve the EPA and DHA content in fish flesh:

1. By finding new genotypes able to conserve higher levels of *n*-3 LC-PUFA. A recent study measured the heritability and inferred mechanisms determining flesh *n*-3 LC-PUFA content in Atlantic salmon (Leaver et al., 2011). The results show that flesh *n*-3 LC-PUFA composition is a highly heritable trait ($h2 = 0.77 \pm 0.14$). This study paves the way for identification of quantitative trait loci and gene interaction networks that are associated with flesh *n*-3 LC-PUFA composition. Research of new genotypes associated with high capacity of LC-PUFA synthesis is a promising way for the future.
2. By creating fish able to produce more EPA and DHA from C18 *n*-3 precursors with the transgenesis. On a model fish species (zebrafish), the EPA/DHA biosynthesis pathway was modified by overexpression of masu salmon (*Oncorhynchus masou*) $\Delta 6$ and $\Delta 5$-desaturase-like gene to increase its ability to synthesize EPA and DHA (Alimuddin et al., 2005, 2007). The results demonstrated that masu salmon desaturase are functional in zebrafish and can modify its fatty acid metabolic pathway. These data suggested that this molecular technique could be applied to farmed fish to generate a nutritionally richer product for human consumption but, of course, after improvement (levels of *n*-3 PUFA produced is always relatively low) and after acceptability by the society.

To our knowledge, the presence of EPA and DHA in diets is still necessary to produce high-quality flesh fish (rich in *n*-3 PUFAS) for human food. This is a very important challenge essential for aquaculture, which needs further studies in fatty acid biosynthesis, especially at molecular levels.

Lipogenesis

In mammals, two key enzymes that are required to divert glycolytic carbon flux into lipid biosynthesis are ATP-citrate lyase (ACLY) and fatty acid synthase (FAS). ACLY converts cytosolic citrate into acetyl-CoA and oxaloacetate, thereby supplying the essential metabolite for lipid biosynthesis. By the action of its seven active sites, FAS catalyzes all of the reaction steps involved in the conversion of acetyl-CoA and malonyl-CoA supplied by acetyl-CoA carboxylase to palmitate. For these reactions, FAS uses NADPH, generated by enzymes of the pentose phosphate shunt such as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), but also by the malic enzyme (ME), and isocitrate dehydrogenase (ICDH; Figure 6.1). Eventually, the whole pool of NADPH will be used as reducing equivalent power for synthesis of palmitate. Under lipogenic conditions, FAS is highly expressed in lipogenic tissues, namely, liver and adipose tissue.

In mammals, FAS expression is exquisitely sensitive to nutritional and hormonal variations. For example, the rate of FAS gene expression is induced by refeeding in rats (Paulauskis and Sul, 1988). Similarly, refeeding highly increased FAS and G6PDH gene expression in rainbow trout liver (Lansard et al., 2009; Skiba-Cassy et al., 2009). The effect of the diet composition on lipogenic gene

expression has also been investigated in fish. While neither dietary energy content nor the presence of carbohydrates in trout diet modified FAS gene expression (Kolditz et al., 2008; Panserat et al., 2009), modification of the fatty acids profile of the diet through fish oil substitution by vegetal oils seems to decrease the capacity of fatty acid biosynthesis indicated by the reduction of FAS gene expression in fish fed vegetal oils (Panserat et al., 2008). Interestingly, low energy-fed trout showed higher gene expression of ICDH in both liver and muscle (Kolditz et al., 2008).

In mammals, insulin activation of Akt induces expression of a number of lipogenic genes, including ACLY and FAS (Porstmann et al., 2005). Several experiments were performed in rainbow trout to investigate the role of insulin in the regulation of lipogenic gene expression and indicated that the effect of insulin may depend on the dose of insulin used and on the way of administration. When trout were acutely treated with a pharmacological dose of insulin, or infused for 5 days with physiological amounts of insulin, hepatic expression of FAS was not modified (Plagnes-Juan et al., 2008; Polakof et al., 2011). Inversely, acute intraperitoneal administration of a physiological dose of insulin increased FAS gene expression in the liver (Polakof et al., 2010a). These discrepancies should probably be related to the regulation of plasma glucose level under insulin treatment. Actually, insulin regulation of FAS transcription is mediated by the PI3 kinase/Akt signaling pathway (Sul et al., 2000). In rainbow trout, like in mammals, glucose have a synergic effect on the insulin stimulation of FAS gene expression as demonstrated in both in vitro (Plagnes-Juan et al., 2008) and in vivo studies (Polakof et al., 2010b). Therefore, lowering plasma glucose levels with insulin administration has probably limited the positive effect of insulin on FAS gene expression.

Important knowledge on the regulation of lipogenic gene expression comes from in vitro studies in which amino acids can potentialize the positive effect of insulin of FAS and ACLY gene expression. This positive effect of insulin and amino acids has been shown to be dependent on the activation of TOR pathway (Lansard et al., 2009, 2010b), suggesting that the regulation of lipogenic genes by the TOR pathway has been conserved between species (Porstmann et al., 2008). The link between the expression of the lipogenic genes and the activation of the TOR pathway has also been suggested by in vivo study. Actually, rainbow trout divergently selected for muscle fat content exhibited not only enhanced expression of lipogenic genes (FAS, ACLY, and G6PDH) but also increased expression of the TOR protein and higher post-prandial activation of the TOR pathway (Skiba-Cassy et al., 2009). Recent findings have indicated that activation of mTORC1 contributes to the regulation of fatty acid biosynthesis by favoring nuclear accumulation of the mature form of the sterol responsive element binding protein (SREBP1) and expression of SREBP target genes such as FAS and ACLY (Porstmann et al., 2008). In rainbow trout, insulin and amino acids regulate SREBP1 gene expression in the same way as lipogenic genes (Lansard et al., 2010b; Polakof et al., 2010b), but the involvement of SREBP1 in the regulation of expression lipogenic genes remains to be established in fish.

As discussed in this review, the regulation of expression of genes related to carbohydrate and lipid metabolism in trout is complex. As demonstrated in mammals, insulin represents a major regulator of metabolic gene expression in fish. However, there is increasing evidence of the involvement of nutrients (amino acids, glucose, and fatty acids) in the regulation of metabolism at the transcriptional level. Thus, metabolic tissues as liver and muscle need to integrate both endocrine and nutritional signals to coordinately control metabolism.

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Chapter 7
Genomic Responses to Stress Challenges in Fish

Lluis Tort and Mariana Teles

Abstract: In this chapter, the genomic responses to stressors related to fish and aquaculture are reviewed. Stress is a common situation that all animals experience and involves a wide array of responses from gene expression to physiological and behavioral responses. The physiological and metabolic responses to stressors have been well documented, but it is only in the last few years that the genomic responses have been assessed. In terms of stressors, the most common husbandry stressors in aquaculture include handling, confinement, or crowding. Moreover, some environmental stressors such as temperature, salinity, and hypoxia can affect both wild and farmed fish. The first part of this chapter will describe gene expression responses of specific genes following the exposure to stressors. In the second part of the chapter, the overall genomic scale responses, analyzed through microarray techniques, are reviewed.

The Nature of the Stress Response

The stress response, meaning the array of changes in different physiological compartments after a symbolic or real threat, and the consequent reaction trying to counteract the effect of the stressor and recover the homeostatic equilibrium, is a common trait in all kinds of animals along the phylogenetic tree. Since all animals may be subjected to stressors and may therefore experience stress, both common and specific responses have been developed through evolution. Some of the common responses are simple ones such as the fight or flight reaction; others relevant to particular neuroendocrine mechanisms or behavioral traits may be specific of a group or a species. Nevertheless, all animals have a repertoire of mechanisms to cope with stressors and to compensate for their effects.

The nature of the stress response is a complex one, and such a response involves many physiological mechanisms, from neural centers and chemical mediators to a wide range of metabolic adjustments. These also involve the active role of the three regulatory systems, neural, endocrine, and immune; thus, generating a wide array of changes in many compartments. Consequences of the first reaction on other systems such as the altered hormonal levels, metabolic, and energetic adjustments, changes in osmoregulation or immune suppression, are commonly seen after most stressors. Since the purpose of these responses is to adapt the physiological systems to the new situation and to organize the metabolism in order to provide the energetic resources required by the stressor, a wide number of metabolic pathways will be involved. Therefore, the stress response will include a very significant number of genes in the reaction in several organs and systems.

The surge of genomic technologies enabled to study the overall gene expression under changing experimental or environmental

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circumstances. Thus, functional genomic technology has been applied in a number of experiments in order to know the response of the genes that are known to be involved in a particular condition and also to see the patterns of activation or inactivation of the different processes and even to discover new genes that appear to be particularly involved in these processes as they may show very significant up- or down-regulation. In this way, one of the processes in which the genomic approach may be most appropriate to check the overall physiology of the animal is in fact the stress response, precisely due to the characteristics of being a holistic response, involving many organs, tissues, systems, and molecules.

Several works have looked at the response of the genome under different kinds of stressors and some of them have dealt with the functional genomic response to stress in fish (Douglas, 2006; Prunet et al., 2008). These studies identified new genes whose expression is significantly modified after exposure to stressors, described processes that are affected by stressors either by activation or suppression, and revealed both common and tissue-specific gene expression signatures. Such studies highlighted the complexity of transcriptional regulation of stress biology (Prunet et al., 2008).

Genomic Approaches to Evaluate the Stress Response in Fish

The response of fish to stress is typically evaluated by measuring levels of hormonal, haematological, and hydromineral parameters in the blood (Barton, 2002; Tort, 2010). Besides the reliability of these traditional stress biomarkers, their use in some circumstances cause some difficulties in the interpretation of results because the response to stress is subjected to modulation by several factors, both intrinsic and extrinsic to the fish (Barton, 2002; Pottinger, 2007). For example, the measurement of plasma cortisol, the main glucocorticoid secreted by the interrenal tissue of the teleosts in response to stress and the most used indication of a stress status, may not be sufficient to assess physiological conditions under chronic stress, in particular due to the acclimation of the interrenal gland and the influence of negative feedback mechanisms on the hypothalamus–pituitary–interrenal axis (Rotllant et al., 2000). Thus, for an accurate assessment of the stress response in aquacultured fish, genomic tools must be combined with the already well-established stress indicators, in an integrated approach, as they help to understand the mechanisms that underlie short-term and long-term environmental adaptations, giving a more complete scenario of the fish condition. The search for the appropriate candidate genes to be included in the set of responses to study can be approached by determining among genes whose expression could reasonably be modified by the different farming conditions (Table 7.1). The stress situations may alter the expression patterns of selected genes that are often unique signatures of a specific stressor. Additionally, gene expression analysis has the potential to precede early signs of injury and be predictive of adverse events.

The present chapter will consider the contribution of selected studies using genomic approaches that have helped the understanding of the fish response to the stress caused by the farming conditions or related to environmental variables (Table 7.2) relevant to aquaculture. These studies that, in general, measure the expression of genes related to the stress response might provide a useful indication of the candidate genes that can be used as biomarkers to diagnose stress and improve stress resistance and fish welfare in the future.

We will focus on two types of functional genomic approaches: First, the gene discovery or gene indication approach that identifies specific genes as indicators of a stressed status. Therefore, such studies focused on the expression of individual genes. Second, the holistic approach using microarrays that searched for molecular signatures and gene patterns following stress

Table 7.1 Studies concerning the effects of husbandry stressors on the expression of individual genes.

Type of stress	Species	Studied organs	Target genes	Main effect	Reference
Transport (acute)	*Dicentrarchus labrax*	Skin, muscle, liver, brain, gills, kidney, gonads, heart, spleen	HSP70	Upregulation in skin and skeletal muscle	Poltronieri et al., 2007
Handling (acute)	*Solea solea*	Ovary and brain	POMC, CB1A, CB1B	Upregulation of POMC. Downregulation of CB1B receptor	Palermo et al., 2008
Handling (acute)	*Acipenser transmontanus*	Yellow corpuscles	StAR	Unaltered	Kusakabe et al., 2009
Handling (acute)	*Perca fluviatilis*	Spleen	GR, MR, lysozyme, hepcidin, chemotaxin, complement unit 3, apolipoprotein A1 and 14 kDa	Upregulation of MR. Downregulation of lysozyme and hepcidin	Milla et al., 2010
Confinement (chronic)	*Sparus aurata*	Brain	S-enolase	Unaltered	Ribas et al., 2004
Confinement (chronic)	*D. labrax*	Liver, brain	Six differentially expressed bands	Upregulation of 4 bands Downregulation of 2 bands	Gornati et al., 2004
Confinement (chronic)	*D. labrax*	Liver	HMGCR, HSP70, HSP90	Upregulation of HMGCR and HSP70	Gornati et al., 2005
Confinement (chronic)	*D. labrax*	Liver, brain	HSP70, HSP90, MT, CYP4501A	Upregulation of HSP70, MT, CYP4501A in liver. Upregulation of HSP70, HSP90, MT and CYP4501A in brain	Gornati et al., 2004

(*Continued*)

Table 7.1 (*Continued*)

Type of stress	Species	Studied organs	Target genes	Main effect	Reference
Confinement (chronic)	*D. labrax*	Liver	GR	Downregulation	Terova et al., 2005
Confinement (acute)	*Gadus morhua*	Blood	Glucose transporters (GLUT1, 2 and 4), G3PDH, SOD, catalase, GSH-Px	Upregulation of all genes, except GLUT1 and GLUT2	Caipang et al., 2008
Confinement (acute and chronic)	*S. aurata*	Liver, brain, head kidney, gills	Glucose regulated protein (GRP75)	Upregulation in liver	Bermejo-Nogales et al., 2008
Confinement (acute and chronic)	*Oncorhynchus mykiss*	Liver	Acute phase response-related genes	Upregulation of SAA, haptoglobin, and DRTP1	Talbot et al., 2009
Confinement (acute)	*D. labrax*	Liver, brain	BDNF	Upregulation of proBDNF and dowregulation of matureBDNF in brain	Tognoli et al., 2010

Table 7.2 Summary of the studies concerning the effects of environmental stressors on the expression of individual genes.

Type of stress	Species	Studied organs	Target genes	Main effect	Reference
Hypoxia (acute)	*Ictalurus punctatus*	Pituitary	POMC	Upregulation	Karsi et al., 2005
Hypoxia (chronic)	*Gadus morhua*	Liver	MT, CYP1A, SOD, catalase, GSH-Px	Upregulation of CYP1A and GSH-Px	Olsvik et al., 2006
Hypoxia (chronic)	*G. morhua*	Gills, heart, liver, white muscle	COX1, Mb, GLUTs, Hks	Upregulation of COX1 and Mb in gills	Hall et al., 2009
Hypoxia (acute and chronic)	*Dicentrarchus labrax*	Liver	HIF-1α	Upregulation	Terova et al., 2008
Hypoxia (acute and chronic)	*D. labrax*	Liver	GLUT2	Upregulation	Terova et al., 2009
Temperature change (acute)	*Danio rerio*	Gonads, gills, liver	HSF1a, HSF1b	Differentially expression	Råbergh et al., 2000
Temperature change (acute)	*Oncorhynchus mykiss*	Blood, brain, heart, liver, muscle	HSP70, HSP30	Upregulation in all organs, except HSP30 in blood	Currie et al., 2000
Temperature change (chronic)	*G. morhua*	Blood	IgM-H, IgM-L, IL-1β, β2-M, MHC class I	Upregulation of all genes except IgM-H	Pérez-Casanova et al., 2008
Salinity change (chronic)	*Sparus sarba*	Gills, kidney, liver, pituitary	$Na^{+/}K^{+}$-ATPase α- and β-subunit, HSP70, HSC70, HSF1, GH, IGF1	Upregulation of $Na^{+/}K^{+}$-ATPase β in kidney and of all HSP70 family genes in gills of seawater and hypersaline-adapted fish	Deane and Woo, 2004
Salinity change (acute)	*Oreochromis mossambicus*	Gills	OSTF1, TFIIB	Upregulation	Fiol and Kültz, 2005
Salinity change (acute)	*O. mykiss*	Gills, liver	$Na^{+/}K^{+}$-ATPase, CFTR, PEPCK, GR	Upregulation of $Na^{+/}K^{+}$-ATPase α in gills, upregulation of PEPCK in liver, upregulation of GR in gills	Singer et al., 2007
Salinity and temperature change (acute)	*Salmo salar*	Gills, kidney, heart, liver, brain	SGRP	Upregulation in gills after hyperosmotic challenge	Pan et al., 2004
Salinity and temperature change (acute)	*Acanthopagrus schlegeli*	Liver	SOD, catalase, GSH-Px	Upregulation after hypo-osmotic and thermal stress	An et al., 2010

treatments, i.e., global gene expression profiling via microarrays.

Studies on Individual Genes

Genomic Responses to Model Husbandry Stressors

Transport and Handling

The effects of the transport and handling stress at the molecular level are among the most common approaches that have been conducted in the last years when looking at husbandry stressors (Table 7.1). Thus, Poltronieri et al. (2007) found an upregulation in the expression of the inducible form of the heat shock protein 70 (HSP70) gene in skin and skeletal muscle of *Dicentrarchus labrax* (European sea bass) subjected to transport stress, whereas no changes were detected in HSP70 expression in the other studied organs (liver, brain, gills, kidney, gonads, heart, and spleen). In a subsequent study with female common sole (*Solea solea*), the expression of genes related with the cannabinergic system were evaluated, because this system is involved in the response to environmental modifications and modulation of adaptation mechanisms (Palermo et al., 2008). In this study, when fish were stressed by handling (catching, netting, and hand-sorting for 1 minute and left undisturbed until sampled), it was found that there is an upregulation of the proopiomelanocortin (POMC) gene in the ovary of stressed fish when compared with controls. On the other hand, the cannabinoid receptor CB1B was downregulated in the ovary of fish 1 and 12 hours after stress, whereas no changes were found 24 hours after handling stress. The mRNA levels of CB1A were not altered during the study. Similar results were found in the brain. According to the authors, the elevation of CB1B receptor expression during nonstressed conditions and its reduction during stress confirm the role of the CB1 receptor as an inhibitor of the synaptic activity and suggest a role of the endocannabinoid system in stress responses in terms of adaptation to environmental conditions.

White sturgeon (*Acipenser transmontanus*) is a primitive bony fish, recognized as an important emerging species for aquaculture. Kusakabe et al. (2009) performed an experiment where fish were stressed by handling, holding them in a net and exposed to air for 30 minutes, and the expression of the steroidogenic acute regulatory protein (StAR) was studied, due to its important and limiting role in the steroidogenic pathway. However, despite the significant increase in plasma cortisol levels, no changes were detected in StAR gene expression in the yellow corpuscles found throughout the kidney. Similar results were obtained by Castillo et al. (2008), as no changes were detected after acute stress. However, significant increases of expression were detected after chronic stress and an even higher response after an immune (lipopolisaccharide injection) stimuli. More recently, a similar experiment was carried out with Eurasian perch, *Perca fluviatilis* by Milla and coworkers. Fish were stressed by acute handling and the expression of several immune-related genes, namely lysozyme, complement unit 3, apolipoprotein A1 and 14 kDa, hepcidin, and chemotaxin, as well as stress-related genes, namely the glucocorticoid receptor 1 and 2 (GR1 and GR2) and the mineralocorticoid receptor (MR), were evaluated in spleen. Despite the increase in plasma cortisol, the GR expression was unaltered in spleen, whereas the MR gene was upregulated in the same organ. Lysozyme and hepcidin were downregulated, while the other studied genes were not affected by handling stress. These authors conclude that in this fish species, acute stress by handling induce alterations of spleen immune defense without any effect on plasma immune parameters and that cortisol/GR would not be a major endocrine regulator of that response.

Confinement and Crowding

Literature is available regarding the effects of stocking density on specific target genes, as the

chronic effects may be of high importance in the aquaculture environment and the consequent implications in performance and profits. One of the first studies concerning this research topic was undertaken in *Sparus aurata* (gilthead seabream; Ribas et al., 2004). In that study, the authors tested the effects of chronic high-density confinement (14 days at 26 kg/m^3 compared with 5 kg/m^3) on the expression of a gene in the fish brain that has a key role in glycolysis, the *S*-enolase gene. Changes were found in the expression of this gene in the brain and also in other sea bream tissues. Other stressors were tested, also giving significant changes, in particular after in vivo LPS injection. These results allowed the authors to suggest that enolase could be one of the potential marker genes for the stress response in fish under diverse conditions, including population density and infection. In a similar study, *D. labrax* were reared at different stocking densities (<10, 80, and 100 kg/m^3) and several genes were found to be differentially expressed (repressed or enhanced) in liver and brain, giving evidence that high densities are responsible for changes in gene expression in specific fish organs (Gornati et al., 2004). Taking into account these findings, the authors cloned the mRNA corresponding to one of those differentially expressed bands, which was identified to be the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase gene (HMGCR), a key enzyme of the cholesterol synthesis (Gornati et al., 2005). Additionally, they evaluated its expression in liver of sea bass reared at different stocking densities and compared with mRNA levels of HSP70 and HSP90. The HMGCR and HSP70 genes were upregulated in the liver of fish reared at high rearing densities, while HSP90 gene expression was unaltered. The authors suggest that HMGCR mRNA could be a good biomarker for detecting fish welfare due to its role in the synthesis of cholesterol, since cholesterol is the substrate for cortisol production and because expression of such a gene resulted in an increase after crowding stress. In a following study of the same research group, the effects of chronic confinement at high stocking densities (80 and 100 kg/m^3) were also evaluated in the liver and brain of *D. labrax* (Gornati et al., 2004), and the analysis of gene expression revealed that in the liver, metallothionein (MT) and CYP4501A genes were upregulated at 80 and 100 kg/m^3, whereas HSP70 were upregulated only at the 100 kg/m^3 and HSP90 gene expression was unaltered. In brain, MT and HSP90 were induced already at 80 kg/m^3; while CYP4501A and HSP70 were affected only by the higher population density. According to the authors, these proteins represent good biomarkers to evaluate fish welfare. More studies were performed with *D. labrax* reared at different stocking densities, a fish that is considered to be a stress-susceptible species. Thus, Terova et al. (2005) demonstrated that chronic stress by high rearing density affects the GR gene expression in liver, whose levels decreased inversely with the increase in plasma cortisol levels. The authors point out GR as another candidate biomarker of a generalized stress response in aquacultured fish.

Concerning other fish species, Caipang et al. (2008) evaluated the effect of short-term overcrowding on the transcriptional profiles of selected glucose transport and antioxidant-related genes in the blood of *Gadus morhua* (Atlantic cod). The authors obtained an upregulation of the glucose transport-4 (GLUT4) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes 2 and 24 hours after crowding. Catalase, glutathione peroxidase (GSH-Px), and Cu/Zn superoxide dismutase (SOD) genes were also upregulated after 2 hours, but thereafter returned to their pre-crowding levels with the exception of SOD that remained significantly higher than the initial group until 72 hours. The authors concluded that short-term overcrowding of Atlantic cod resulted in enhanced transcriptional activity of glucose transport and antioxidant defense genes and that the antioxidants SOD could be used as a good biomarker for oxidative stress resulting from overcrowding, since this gene was upregulated during all the exposure trials.

Bermejo-Nogales et al. (2008) evaluated the tissue-specific regulation of glucose-regulated protein 75 (GRP75), a molecular chaperone, in *S. aurata* subjected to an acute confinement exposure (24 hours at 120 kg/m^3) and to prolonged confinement exposure (3 weeks at 120 kg/m^3). After the acute confinement, fish exhibited an upregulation in liver GRP75 mRNA levels. However, in head kidney, brain, and gills, acute confinement exposure did not induce any effect in the expression of this gene. The chronic confinement also caused an upregulation of GRP75 in liver, whereas transcript levels of GRP75 in brain, head kidney, and gills were unaltered. The authors concluded that hepatic transcription of GRP75 was altered by acute and chronic confinement stress, whereas extrahepatic tissues were apparently refractory though not excluding the possible masking effects of different tissue kinetics.

Differential gene expression was investigated to identify acute phase proteins induced by confinement stress in the liver of rainbow trout (*Oncorhynchus mykiss*), providing a method of assessing the activation of the neuroendocrine stress response (Talbot et al., 2009). The results showed that from the eight selected genes, the expression of three genes, namely serum amyloid A (SAA), haptoglobin, and differentially regulated rainbow trout protein 1 (DRTP1), were the most differentially expressed gene as a result of exposure to confinement stress. The increase of haptoglobin and DRTP1 gene expression and its uniformity in response to stress make these genes suitable biomarkers for stress in rainbow trout. In addition, it was intended to assess the variation in gene expression between individual fish within the population of stressed fish in an attempt to identify acute phase proteins having uniform and consistent changes in expression during stress. When the gene expression was analyzed among individual fish, it was found that the transcript levels of haptoglobin and DRTP1 genes varied much less between individuals than was the case for SAA.

All the cited studies deal with the typical stress-related genes, such as MT, HSP, GR, and glucose transporters. However, Tognoli et al. (2010) searched for alternative molecular biomarkers of stress. Thus, they investigated the effects of an acute stress on the expression of a neurobiological marker, the brain-derived neurotrophic factor (BDNF) in the brain of sea bass. This gene has recently been involved in stress-induced adaptation as a key regulator of neuronal plasticity and adaptive processes in mammals. The authors concluded that in sea bass, the BDNF gene is expressed in neuronal and nonneuronal tissues and that the proBDNF/totalBDNF ratio is a novel quantitative biomarker capable to detect biological stress in sea bass.

Genomic Responses to Environmental Stressors

Hypoxia

Hypoxia is an important stress factor for all fish species and was among the first stressors to be analyzed using a genomic approach. Channel catfish (*Ictalurus punctatus*) was subjected to an acute low-water stressor, and the authors (Karsi et al., 2005) studied the expression patterns of the POMC gene in the pituitary, since this gene plays an important role in the hypothalamic–pituitary–adrenal axis being a precursor of several peptide hormones, including adrenocorticotropic hormone, melanocyte-stimulating hormones, and β-endorphin. POMC gene was upregulated 1 hour after recovery; however, 3 hours after recovery, POMC mRNA expression levels declined to the levels of control group. Plasma cortisol levels were elevated during the stress application and returned to normal during recovery. The authors conclude that POMC gene and cortisol are both involved in the low-water stress response during which cortisol may serve as a negative regulator of POMC expression in catfish. Afterward, Olsvik et al. (2006)

evaluated the effect of chronic hyperoxia or hypoxia on the transcription of genes related to stress and to the oxidative stress status in the liver of the Atlantic cod. Thus, after 6 weeks of hypoxia, GSH-Px, SOD, and cytochrome P401A (CYP1A) expression was downregulated in the liver of fish, suggesting a suppression of the tissue activity with reduced cell turnover and proliferation of liver cells. With the same species, the authors evaluated the expression level of genes associated with oxygen (cytochrome oxidase 1 (COX1) and myoglobin (Mb)) and glucose utilization (glucose transporters (GLUTs) and hexokinases (HKs)) in fish subjected to an hypoxic challenge for 24 days (Hall et al., 2009). Over the first 6 days, increased average expression levels of GLUTs and HKs in gill, heart, liver, and white muscle in hypoxic fish was detected. There were significant increases in COX1 and Mb expression levels in gill by day 24, but no changes in these aerobic indicators in heart or liver. Long-term hypoxic exposure of Atlantic cod results in increases in gill COX1 and Mb mRNA, while increases in gene expression related to glucose mobilization (GLUT2), uptake (GLUT1 and GLUT4), and metabolism (HKI) are short-lived. The authors concluded that the data suggest a transient increase in genes associated with glucose utilization during the early part of the hypoxic challenge followed by alterations in gene expression in gills.

Terova and collaborators also evaluated the effects of both acute and chronic hypoxia in the expression of genes in sea bass liver. In the first study, the authors stated that acute and chronic hypoxia upregulated the hypoxia-inducible factor-1 (HIF-1α) transcription in liver; however, these changes were transient and returned to normal levels upon reexposure to normoxic conditions (Terova et al., 2008). In the second study, they evaluated the expression of the sodium-independent glucose transporter (GLUT2) in liver in response to hypoxia. The authors found an increased mRNA expression of the GLUT2 gene in response to both acute and chronic hypoxia, supporting the view that GLUT2 is involved in the adaptation response to hypoxia in sea bass, a marine hypoxia-sensitive species (Terova et al., 2009).

Temperature Change

Aquacultured fish can be exposed to water temperatures that do not correspond to their thermal preferences, since their movement in the water column can be limited due to particular farming circumstances (Gollock et al., 2006). Given that temperature has a considerable influence in biochemistry, physiology, and behavior (Dominguez et al., 2004), elevated or decreased temperatures can have a negative effect on fish health. However, over the last decade, only few studies have deal with the effects of temperature change on the expression of specific genes in fish. The transcriptional regulation of HSP genes is mediated by the heat shock transcription factor (HSF). This reason motivated Råbergh et al. (2000) to clone the first HSF from fish, zebrafish *Danio rerio*. The analysis by reverse transcriptase polymerase chain reaction (RT-PCR) revealed the presence of two HSF1 mRNA forms that were expressed in a tissue-specific dependent manner in response to heat stress. Both forms were expressed in gonads under all conditions; in liver and to a lesser extent in the gills, the longer splice form of HSF1 disappears upon heat shock, revealing a tissue-specific regulation of HSF1 upon exposure to elevated temperature. Heat stress also induced an upregulation of HSP70 in several organs (blood, brain, heart, liver, and muscle) of rainbow trout. In addition, HSP30 mRNA was upregulated in almost all organs (except blood; Currie et al., 2000). The authors concluded that increases in HSP70 mRNA levels in blood may serve as an early indicator of temperature stress in fish; however, the tissue type, thermal history, as well as the particular family of HSP must be considered when evaluating the thermal stress using these molecular approaches. In a latter study evaluating the effect of the thermal stress in fish, Atlantic cod was exposed to a thermal challenge, and the blood

expression of some relevant immune genes were assessed (Pérez-Casanova et al., 2008). The expression of immunoglobulin M heavy chain (IgM-H) remained unaltered during the experiment. Though, interleukin-1β (IL-1β) gene was upregulated at 19°C (the highest tested temperature that is near the cod's upper thermal limit), and changes in the expression of β2-microglobulin (β2-M), major histocompatibility complex class I (MHC class I), and IgM-L followed a pattern similar to that seen for plasma cortisol, i.e., an increase at 16°C, and a return to pre-stress levels at 19°C, revealing a biphasic response that could be explained by cortisol modulatory effects or the presence of an optimal temperature for immune function in this species.

Salinity Change

Various anatomical and physiological changes allow the successful transition of migratory fish, such as eels or salmons, from freshwater to seawater, a transition that may be considered a salinity stress. In this perspective, Deane and Woo (2004) evaluated the expression of seven key genes involved in the ion regulation (Na^+/K^+-ATPase), cytoprotection (HSP70), and growth (somatotropic axis) in silver seabream (*Sparus sarba*) adapted to hypo-osmotic, iso-osmotic, seawater, and hyperosmotic environments. In gills, the transcriptional expression profile of Na^+/K^+-ATPase α- and β-subunit genes were lowest in iso-osmotic-adapted fish, whereas in kidneys, the expression of the β-subunit increased in seawater and hypersaline adapted groups. The HSP70 multigene family, comprising genes coding for heat shock constitutive (HSC70), inducible heat shock protein (HSP70), and a heat shock transcription factor (HSF1), was found to be highly upregulated in gills of seawater and hypersaline-adapted fish. In liver, HSC70 expression was lowest in iso-osmotic groups, and in kidneys, the HSP70 multigene family remained unchanged over the salinity range tested. The regulation of the somatotropic axis was studied by measuring pituitary growth hormone (GH) expression and liver IGF1 expression in salinity-adapted fish and reveals that both genes were upregulated in fish maintained at iso-osmotic salinity. With these data, the authors demonstrated how certain processes are modulated in a euryhaline fish during salinity adaptation, providing new information on key molecular processes involved in fish euryhalinity. In a following work, tilapia (*Oreochromis mossambicus*) were acclimatized from freshwater to seawater (hyperosmotic environment), and two transcription factors, the osmotic stress transcription factor 1 (OSTF1) and the tilapia homolog of transcription factor II B (TFIIB), were identified by suppression subtractive hybridization (SSH) of gill mRNAs (Fiol and Kültz, 2005). These transcription factors were rapidly and transiently induced during hyperosmotic stress, and they increased gradually with increasing salinity. The authors conclude that OSTF1 and TFIIB seem to be key elements for transcriptional regulation of transport proteins and gill cell differentiation during osmotic adaptation.

The effects of hyperosmotic stress at the transcriptional level were also evaluated in rainbow trout by Singer et al. (2007). They assessed the expression patterns of GR in the liver and gills of fish during gradual salinity acclimation. Thus, the gill GR expression was upregulated upon salinity exposure, and this level was maintained over the 5-day period, while liver GR transcript levels were unaltered. With these findings, the authors demonstrated a tissue-specific GR expression associated with salinity acclimation and a role for osmosensory signal transduction pathway in the regulation of GR expression in fish. Additionally, changes in the transcript levels of the gill Na^+/K^+-ATPase and cystic fibrosis transmembrane conductance regulator (CFTR), as well as liver phosphoenolpyruvate carboxykinase (PEPCK), were also evaluated, as indicators of salinity-mediated cortisol signaling in trout gill and liver, respectively. Thus, while gill Na^+/K^+-ATPase α-subunit mRNA showed a transient upregulation with salinity exposure, gill CFTR mRNA was not affected, leading the

authors to hypothesize that CFTR transcriptional induction may require a higher salinity threshold unlike the sodium pump or that the higher cortisol response, evident with abrupt transfer studies but absent in this study, may have a permissive role in the regulation of CFTR expression in fish. The transient upregulation of sodium pump transcripts to salinity exposure may be critical for the maintenance of elevated gill Na^+/K^+-ATPase activity over the salinity acclimation period (Shepherd et al., 2005). Liver PEPCK was also transiently upregulated at day 1, but not at day 3 or day 5 of salinity exposure.

Atlantic salmon (*Salmo salar*) exposed to hyperosmotic conditions had an upregulated transcript in branchial lamellae, which was found using differential assay of mRNA expression (Pan et al., 2004). This transcript corresponded to a salmon glycine-rich RNA-binding protein (SGRP) and possessed a high degree of identity with the cold-inducible RNA-binding proteins of mammals and amphibians. SGRP transcript was also observed in liver, kidney, and heart, but was not altered by osmotic stress in these tissues. SGRP expression in the different salmon organs was also unaltered after exposure to cold. The authors suggest that SGRP appears to have a more prominent role in adaptation to hyperosmotic conditions rather than to cold stress. Finally, An et al. (2010) determined oxidative stress by measuring the expression of three antioxidant enzymes, namely SOD, catalase, and GSH-Px in black porgy (*Acanthopagrus schlegeli*) exposed to thermal or hypo-osmotic stresses. The three genes were upregulated after both exposure challenges, indicating thermal and hypo-osmotic stress are able to cause an oxidative stress in this fish species, suggesting that black porgy have the antioxidant system against stress factors.

Microarray-Based Studies

While the analysis of individual genes has provided useful information, genomic screening approaches using microarray technology have only recently been applied to the study of stress responses in fish. These new screening methods potentially provide a system-wide assessment of stress-induced alterations in gene transcription, which should not only generate overviews of the biological pathways and processes involved in the stress response, but also provide the means to identify new candidate genes (Cossins et al., 2006). These strategies are based on the assumption that data generated by a genomic approach provides a more comprehensive view of the complexity of the responses to stressors.

The use of microarrays for the study of various aspects of fish physiology has seen a spectacular increase in the most recent years. From early studies with model species, such as zebrafish, to current studies with commercially important species, such as salmonids, catfish, carp, and flatfish, microarray technology has emerged as a key tool for understanding developmental processes as well as basic physiology. A number of different platforms are now available, ranging from microarrays containing cDNA amplicons to oligomers of various sizes. High-density microarrays containing hundreds of thousands of distinct oligomers have been developed for zebrafish and catfish. As this technology advances, so will our understanding of global gene expression in fish. Furthermore, lessons learned from this experimental group of model organisms can also be eventually applied to other aquaculture organisms.

Genomic Responses to Model Husbandry Stressors

Transport and Handling

Different authors have studied the transcriptomic response after handling. Using the *Stressgenes* platform, Krasnov et al. showed the handling stress effects on gene expression in a microarray after netting rainbow trout for 2 minutes and repeating this procedure once

a day for a duration of 5 days. Samples were collected from brain and kidney after 1, 3, and 5 days, and analysis was performed using Gene Ontology. The results showed that mitochondria, extracellular matrix, and endopeptidases (especially collagenases) were the major targets in kidney. Stress response in brain was characterized by dramatic temporal alterations since some genes were induced transiently (metal ion binding proteins, glycolytic enzymes, and motor proteins), whereas expression of other genes changes behaved in a reciprocal fashion (cell proliferation and growth, signal transduction and apoptosis, protein biosynthesis, and folding). Overall, this work pointed out that the stress responses in fish brain and kidney are different both in function and time-course and that microarray studies also need to perform such a temporal and tissue-specific survey in order to provide precise information for gene expression response to stressors (Krasnov et al., 2005).

Momoda et al. (2007) used an oligonucleotide microarray and real-time RT-PCR to study gene expression in the liver of rainbow trout after netting and handling stress for 3 hours and analyzed the results 0.5, 3, and 21 hours after experiencing the stressor. Most of the genes altered were related to immune-related mechanisms, energetic metabolism, and cell growth and apoptosis. These results also showed that some of the genes related to cell growth were still elevated 21 hours after stress, which indicates that recovery was incomplete at that time. In addition, it was shown that sex differences do exist and that males and females use different cellular/physiological pathways to respond to stress and also for the recovery, regardless of the stage of their sexual maturation (Momoda et al., 2007). Other works have assessed the changes in gene expression by microarray using an in vitro approach with cultured cells treated with an immune stressor (LPS) and compared the transcriptomic response when adding cortisol. The results showed that cortisol is able to counteract the immune activation, but other responses were taking place anyway, such as the recovery of cell activity, increase of protein synthesis, and energetic metabolism (Mackenzie et al., 2006).

A significant number of works have been performed in salmonids but less work has been done in other aquacultured species such as sea bass and sea bream, although some studies are already available in some of these species also concerning stress response. Thus, a cDNA microarray containing 10,176 clones from a cDNA library of mixed embryonic and larval stages was constructed for sea bream (Sarropoulou et al., 2005) and a number of genes were identified related to larval stages. In addition, the same authors assessed the gene expression in juveniles of sea bream injected with a cortisol implant containing 10 mg/kg (fish wet weight) cortisol. The results revealed 257 significantly regulated genes, involving genes related to carbohydrate metabolism as well as genes involved in Na+/K+ transport, polyamine biosynthesis, and iron homeostasis (Sarropoulou et al., 2005).

As most studies on the gene expression in fish have concentrated in the early response, Wiseman et al. (2007) designed an experiment looking at the changes during adaptation after acute stress in rainbow trout. The study was concentrated in the liver as the major target organ for metabolic adjustments in order to perform a selective transcriptomics approach to address the molecular response in this tissue during recovery. The stressor consisted of a standardized 3-minute handling disturbance and liver samples were collected either prior to or 1 and 24 hours after stressor exposure. A low density custom cDNA array consisting of 147 rainbow trout genes was designed specifically, and the acute stress response was characterized by significant upregulation of 40 genes at 1 hour, and 15 genes at 24 hours after stressor exposure in liver. Many of these genes were involved in energy metabolism, implicating a rapid liver molecular reprogramming as critical for the metabolic adjustments to an acute stressor, but also other genes involved in immune function and protein degradative pathways

(Wiseman et al., 2007). These authors also suggest a role for stressor-mediated genomic cortisol signaling in the liver molecular programming associated with stress in fish.

Confinement and Crowding

Cairns et al. (2007) used the Stressgenes platform to study transcriptomic changes after confinement in rainbow trout liver looking at the early (before 48 hours) and late (4–21 days) responses. A total of 314 genes were differentially regulated, mostly comprising genes related to acute phase proteins and involved in the responses to stimuli and extracellular components. Most of the changes were found during 24–168 hours.

More recently, Calduch-Giner et al. (2010) used a cDNA microarray enriched with stress and immunorelevant genes in liver samples to assess the time-course response of seabream after confinement. After detecting 202 genes differentially expressed, the change pattern showed a rapid first phase of metabolic readjustments involving transport of fatty acids for metabolic fuelling, a second phase of repair and remodeling processes, and a last phase encompassing cell homeostasis with increased cell trafficking, involving scavenging reactive oxygen species and regulation of immune system. These results stressed the importance of the role of endoplasmic reticulum for homeostatic adjustments.

A similar analysis was also carried out with the head kidney of rainbow trout exposed to a confinement stressor and revealed changes in biological functions such as protein turnover, energy demand, steroidogenesis, immune response, detoxification, oxygen transport, cellular structure, electron transport chain, signal transduction, and ion transport (Prunet et al., 2008). In these works, the complex nature of the stress response can be visualized from the fact that a number of hormones not previously associated directly with stress in fish, such as insulin-like growth factors, testosterone, and insulin in the liver, and prolactin, GH, and somatolactin in the head kidney (Wiseman et al., 2007; Prunet et al., 2008).

Studies on salmon fry using microarrays showed that handling, confinement, or the overall mannipulation causes a number of alterations that may result in early mortality. In fry with early mortality, all of the responses were compatible with immediate stress, leading rapidly to death, whereas in fry exhibiting intermediate mortality, disturbances were detected in globin gene expression and hypoxia-related genes. In fry that exhibited late mortality, changes in gene expression were mainly associated with reduced metabolism (Vuori et al., 2006).

Other types of stressors that sometimes involve handling and confinement are related to social stress, for instance, confining fish in a set up in which dominant individuals may exert stressful influences in other cohabitants. Sneddon et al. (2005) applied a microarray approach to study gene expression profiles in brain tissue, and about 1165 genes differed between fish of different social status. These differences were mostly related to processes like protein turnover, metabolism, and a number of genes related to stress responses and behavior. Moreover, recent genomic work has also pointed out that the response of stressors may significantly depend on the coping strategy adopted by fish. Thus, although the variability of responses among individuals was known and the relative consistency of the stress responses was assumed (Tort et al., 2001), the genomic analysis showed the importance of individual and group differences inside one species (MacKenzie et al., 2009).

Responses to Environmental Stressors

Identification of differentially expressed genes in response to environmental changes (Table 7.2) offers insights into the roles of the transcriptome in the regulation of physiological responses (Gracey, 2007). Although mild environmental changes may not result in

a stress situation for the fish, more abrupt or sudden changes or the occurrence of slighter but chronic variations induce an altered status and become a stress episode that may generate stress responses (Table 7.2).

Hypoxia

Since one of the major environmental stressors for fish is hypoxia, a number of studies have been carried out using microarray techniques to check the effect of low oxygen levels on the response of the genes. Thus, microarrays are one of the most capable techniques of performing large-scale gene expression screens to investigate the molecular and physiological mechanisms underlying hypoxia, to identify candidate hypoxia biomarkers, and to characterize effects of hypoxia on development and physiology of fish (Ju et al., 2007; Zhang et al., 2009).

Although few in number, some of the existing microarray-based studies have already provided important insights into hypoxia responses. For example, Ton et al. (2003) exposed zebrafish embryos to extreme hypoxia for 24 hours and documented arrested development, together with a series of changes in gene expression consistent with a switch to anaerobic metabolism and downregulation of energy consumption (e.g., via downregulation of protein synthesis and channel arrest). They also demonstrated that hypoxia-induced changes in gene expression revert to normal upon a brief subsequent exposure to normoxia. Van der Meer et al. (2005) focused on long-term adaptive responses to hypoxia and selected the gills of the adult zebrafish as a target tissue. They observed metabolic depression, as indicated by repression of genes in the tricarboxylic acid cycle and protein biosynthesis, and postulated that the induction of genes for lysosomal lipid (e.g., cholesterol) trafficking and degradation represent an adaptive response to hypoxia. Other studies have examined effects of hypoxia on global gene expression in multiple tissues (heart, muscle, liver, brain) in hypoxia-tolerant goby, *Gillichthys mirabilis* (Gracey et al., 2001), and medaka (*Oryzias latipes*). In both studies, the effects of hypoxia varied substantially among tissues and reflected the need for activity maintenance during hypoxia, as well as the extent of the hypoxic insult that each tissue was experiencing. For example, in skeletal muscle, which accounts for approximately one-half of the mass of the organism, the major energy-requiring processes like protein synthesis and locomotion were shut down very rapidly after onset of hypoxia. Then, after 24 hours, a strong induction in the liver of genes needed for enhanced anaerobic ATP production and for gluconeogenesis occurred. Concurrently, cell growth and proliferation were suppressed, which may serve to divert important energy resources away from growth toward those metabolic processes more essential for hypoxia survival (Gracey et al., 2001).

In the work by Ju et al. (2007), a microarray containing 8046 genes measured gene expression changes in the brain, gill, and liver of hypoxia-exposed medaka. The results showed that 501 genes in the brain, 442 in the gill, and 715 in the liver were differentially expressed in hypoxic medaka. These differentially expressed genes fell into a number of biological gene ontology groups related to general metabolism, catabolism, RNA, and protein metabolism, with two biological pathways, ubiquitin–proteasome and phosphatidylinositol signaling, that were significantly dysregulated in medaka upon hypoxia exposure (Zhang et al., 2009).

In adult zebrafish, a cDNA microarray expression study on 15,532 genes of hypoxia-induced changes in the gills identified 367 differentially expressed genes, of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions. Metabolic depression was indicated by repression of genes in the TCA cycle in the electron transport chain and of genes involved in protein biosynthesis (van der Meer et al., 2005). Enhanced expression of the monocarboxylate transporter and of the oxygen transporter Mb was also observed. Further changes in other adaptive mechanisms in

front to hypoxia were identified, such as the induction of genes for lysosomal lipid trafficking and degradation (van der Meer et al., 2005). Martinovic et al. (2009) studied in vivo responses to hypoxia in zebrafish but related to its reproductive system, using the gonads as the target tissue with the objective of characterizing modes of action by which hypoxia could potentially alter reproduction. Thus, zebrafish were subjected to moderate hypoxia (3 mgO^2/L) and severe hypoxia (1 mgO^2/L) for 4 and 14 days, and responses were evaluated using a commercial 21,000 gene zebrafish oligonucleotide microarray. Short-term or longer-term exposure affected different GO categories. Short-term affected expression of genes related with metabolism of carbohydrates and proteins, nucleotide metabolism, haemoglobin synthesis, reactive oxygen species metabolism, and locomotion. Prolonged hypoxia affected the lipid metabolism, reproduction, and immune responses. This study allowed the identification of the expected mechanisms such as modulation of expression of steroidogenic genes and downregulation of serotonergic pathway and also other less evident mechanisms such as reorganization of lipid transport or downregulation of contractile elements (Martinovic et al., 2009).

Zebrafish embryos have also been studied in order to determine the effect of 24 hours of hypoxia during development. Using a 4500 genes zebrafish cDNA microarray, it was demonstrated that hypoxia changed the gene expression profile of the zebrafish embryos and that these changes could be reverted by exposure to a normoxic environment. However, it remained to be confirmed which changes were the direct effects of hypoxia and which were the indirect effects of developmental arrest, thus requiring more research on the molecular pathways specifically responsive to hypoxia (Ton et al., 2003).

Temperature Change

Microarray work on the effects of temperature as stressor in fish has concentrated in sudden increases or decreases of temperature, as chronic changes are more related to seasonal variations and may not represent a stressor for the fish.

Responses across seven tissues assessed by a microarray composed of 13,440 cDNA probes in carps exposed to increasing levels of cold showed a large set of unique cDNAs affected by cold. These cDNAs included an expression signature common to all tissues of 252 upregulated genes involved in RNA processing, translation initiation, mitochondrial metabolism, proteasomal function, and modification of higher-order structures of lipid membranes and chromosomes (Gracey et al., 2004). With this approach, an expression signature suggestive of atrophy in cooled skeletal muscle could be proposed (Gracey et al., 2004). Also, these authors identified large numbers of transcripts with highly tissue-specific patterns of regulation. In a subsequent work on carp subjected to cold, a microarray analysis in several tissues and the biological processes associated with metabolism revealed that the brain is one of the most active tissues upregulating processes during cold-acclimation, for instance, in lipid metabolism, metabolic-energetic changes, or upregulating the tricarboxylic cycle. Translation processes, protein folding genes, and chaperones are also upregulated during cold-acclimation and were also observed to be upregulated in all tissues with the exception of the intestinal mucosa (Gracey, 2007).

Work done in the Atlantic cod used the SSH technique in a cDNA library to detect dysregulated genes after heat shock in liver, skeletal muscle, and head kidney. These tissues were selected due to their roles in metabolic regulation, locomotion and growth, and immune function. HSP90a and HSP70-1 expression showed significant upregulation in all three tissues studied, but they were more than 100-fold upregulated in liver following heat shock. Toll-like receptor 22 transcripts found in the liver were shown to be significantly downregulated in the head kidney after heat shock, which would involve heat-induced immune suppression. In the coral reef fish *Pomacentrus moluccensis,* a

microarray survey on the early transcriptional response to heat stress was undertaken to characterize the response to heat stress by a heterologous microarray approach (zebrafish). A total of 111 gene loci showed significant changes in transcript abundance following exposure to elevated temperatures, many of which were involved in protein processing, transcription, and cell growth (Kassahn et al., 2007). The gene expression response of *P. moluccensis* to heat stress observed in this study indicated suppressed cell growth, the repression of transcriptional activity, and increased protein breakdown. These responses are consistent with a metabolic reorganization following the onset of stress, which would precede the induction of genes and de novo synthesis of proteins, which may ultimately allow the organism to cope with prolonged exposure to stress (Kassahn et al., 2007).

Longer-term studies showing the response to temperature changes have also been performed using microarray techniques. Under these circumstances, one may assume that these experimental fish may be subjected to a mild chronic stress but also that they are developing response in order to acclimate to temperature variation.

Using a cDNA microarray, Logan and Somero (2010) looked at the transcriptional profiles in gill tissue of a highly eurythermal goby fish, following 4 weeks of acclimation to 9°C, 19°C, or 28°C. While gill transcriptomes were not strikingly different, as only 9% of the genes were significantly different in expression among the three acclimation groups, the 59% of the variation in expression among these genes showed an upregulation with increasing acclimation temperature. The most important changes were related to energetic costs of macromolecular turnover and membrane-localized transport. Thus, gene ontology analysis identified protein biosynthesis, transport, and metabolism. In this work, it is relevant to note that no significant upregulation of heat shock proteins or similar stress-related proteins was observed in the long-term 28°C-acclimated fish, showing a process of gradual adaptation to a the new temperature condition (Logan and Somero, 2010). In the same direction of temperature fluctuation, changes in gene expression have been studied in the eurythermal fish, *Austrofundulus limnaeus*, subjected to long-term acclimation to constant temperatures of 20°C, 26°C, and 37°C and to environmentally realistic daily fluctuations in temperature between 20°C and 37°C. Control of cell growth and proliferation appears to be an important part of the response to change in temperature, although these processes appear to be regulated by different genes in constant versus fluctuating temperature regimes, for instance, using small or large molecular chaperones. Other genes involved are the enzymes involved in the biosynthesis in the maintenance of membrane integrity and in fatty acid saturation for fluctuating temperatures and cholesterol metabolism for short-term changes. The genes related with daily rhythms, and especially the genes playing a role in the assembly of transcription initiation, may act as a compensatory modulator of transcription in response to temperature, and thus, it may represent a global gene expression temperature sensor (Podrabsky and Somero, 2004).

A recent work done on rainbow trout looked specifically at the red blood cells subjected to heat shock and showed that genes involved in the stress response, immune response, and apoptosis were among those showing the highest alterations during both early and late transcriptional regulation (Lewis et al., 2010). Using the GRASP salmonid microarray, differences in RBC global gene transcription were investigated in rainbow trout under control conditions (11°C) and exposed to heat stress (1 hour at 25°C followed by recovery at 11°C) and analyzed at 4 h (representing early transcriptional regulation) and 24 hours (late transcriptional regulation). Additionally, genes related to the differentiation and development of blood cells were transcriptionally upregulated at the 24-hour time point. These authors suggest that transcripts that were consistently

dysregulated in blood in response to heat stress are potential candidates of nonlethal biomarkers of exposure to this particular stressor (Lewis et al., 2010).

Salinity Change

Many of the studies on osmoregulation and salinity challenge, including the genomic studies, have been performed in salmon species since they naturally experience such a challenge when changing between freshwater and seawater environments. While this has been the usual interest of researchers, the functional genomics studies have helped to provide additional answers on unknown problems that may face these fishes. Thus, a recent work on mortalities of migrating sockeye salmon (*Oncorhynchus nerka*) has revealed that survival was correlated with a common genomic profile that was correlated with mortality. In ocean-tagged fish, a mortality-related genomic signature was associated with a 13.5-fold greater chance of dying en route. In river-tagged fish, the same genomic signature was associated with a 50% increase in mortality before reaching the spawning grounds, and the same signature was associated with 3.7-fold greater odds of dying without spawning at the spawning grounds. The functional analysis raised the possibility that the mortality-related signature was associated to a viral infection rather to salinity or osmoregulation-related challenges (Miller et al., 2011).

To screen the osmoregulatory tissues using a microarray approach in European eels, a number of 229 differentially expressed clones with unique sequences involved in cellular and/or whole animal osmoregulation were identified. Many of the genes downregulated following seawater acclimation were found in the intestine, as it is known that eels stop feeding in freshwater, and the role of the intestine is limited to osmoregulation during their spawning migration. Thus, many genes known to be involved with digestion and food absorption within the GI tract showed such inhibited expression (Kalujnaia et al., 2007).

A sufficient and timely energy supply is a prerequisite for the operation of iono- and osmoregulatory mechanisms in fish. The functional genomics may also provide another powerful approach to explore new metabolic pathways related to fish ion regulation. In the lists of differentially expressed genes, there are many genes that are relevant to energy metabolism; however, the physiological significance of those energy metabolism-related genes have been overlooked. The regulation of genes associated with many metabolic processes, including the mitochondrion electron transport chain, the TCA cycle, glycolysis, polysaccharide and amino acid catabolism, peptide cleavage and transport, proteolysis, fatty acid catabolism, lipid binding and uptake, and polyunsaturated fatty acid catabolism have been evaluated in several fish species. For different metabolic processes, it was found that regulation is dependent on the organ and the fish species, but also to the degree of salinity, and the duration of acclimation to salinity changes (Tseng and Hwan, 2008).

Conclusions

Despite dramatic differences between tissues and time points, the genomic studies performed up to now have been able to identify a number of genes that researchers propose that can be good, reliable, or suitable indicators of a stress status. The proposed genes include either activators of the brain activity, hormone receptors, acute phase or heat shock proteins, immune modulators, and a number of metabolic mediators. Overall, it is not accurate to conclude that some specific genes will become good indicators for any species, condition, or experimental setup, but rather that individual genes are markers of a particular stressor in a given condition. Nevertheless, it is also true that genes encoding for acute phase responses or brain activation can indicate acute stress and that genes

involved in metabolism should be modified after chronic stress since a wide reorganization of resources should take place to cope with the consequences of this chronic stress.

Besides, groups of genes might show strong correlation of expression profiles. The meta-analysis studies regarding integration of different genomic works are not common, but for instance, Krasnov et al. (2005) showed 48 genes being regulated by stress in 35 different microarray experiments. Overall, the number of differentially expressed genes is markedly higher in EST than in genes selected through Gene Ontology annotations; nevertheless, the identification of stress-regulated genes provide the possibility for analyzing the patterns of the stress responses in various conditions and again a further search for the functionally related genes (Krasnov et al., 2005). These more holistic and global approaches have the advantage of being less biased and more informative with regard to the biological process of interest when compared with a reductionist single-gene/protein approach that requires prior knowledge about which genes/proteins are involved.

Some points of concern are still to be considered, as the sex differences in gene expression, which may be common and should be addressed more directly. Males and females may be utilizing different cellular/physiological pathways to respond to and recover from the same stress, regardless of the stage of their sexual maturation (Momoda et al., 2007). Another area to be explored in future work is the assessment of the effect of stress on specific developmental stages of fish, and as a consequence, the use of whole fry as an effective sample, as several stress-responding genes isolated in early stages have also found to be regulated along later phases of development (Sarropoulou et al., 2005). Another of the problems can be the use of these existing genomic tools or databases in "nonmodel" organisms, as additional technical improvements will be required to further increase success rates of identification. This may be a precise barrier for work with nonmodel species, as the reliance of models of biochemical pathways, gene ontology databases, and other information are largely based on data obtained with model species. Although many biochemical pathways and biological processes are evolutionarily highly conserved, a great amount of plasticity is inherent in cellular metabolic and signal transduction networks (Kültz et al., 2007).

Other shortcomings can be identified, including lack of information on the longer-term compensatory or adaptive phases of the stress response. When transcriptional differences are searched among long-term acclimation groups, they may reflect an acclimation process that has largely remedied the effects of acute stress and established a new steady-state condition involving changes in relative energy costs for different processes. Nevertheless, this pattern of transcriptional alteration in steady-state-acclimated fish may be a signature of adaptation (Logan and Somero, 2010). Limitations have also been quoted regarding the gene annotation and the use of pooled mRNA preparations, which masks variation between individuals (Prunet et al., 2008; MacKenzie et al., 2009), although some of these points will be probably solved in a short time, due to the increasing power of new generation genomic technologies. High-throughput transcriptomics and proteomics technologies are powerful tools for identifying specific gene patterns in an increasing number of species. The *meta* studies performing assessment of gene changes after several treatments of handling and confinement or heat stress have found a number of genes that are consistently dysregulated. In this way, it has repeatedly suggested that when these genes are found in regulatory organs and blood, they may well be potential good candidates for nonlethal biomarkers in aquaculture after exposure to stressors or providing a profile associated with stress.

Enumeration of gene-expression profiles could provide a key for interpreting stress responses in fish groups or populations and for diagnosing specific causes of the

husbandry-related or environmental stress response. Rapidly growing sequence databases are providing genetic tools for dissecting stress responses in many species, and sequences are being more and more available. Together with metabolic, physiologic, and molecular data, this presents a holistic view of the machinery of the cell across several layers of complexity. The study of gene expression modifications at changing environmental conditions can help in understanding stress responses and also furnish fast and reliable molecular biomarkers to diagnose the status of the animal. A particular strength of the genomic techniques is that it is relatively simple nowadays to construct a microarray for any organism, and access to the equipment needed to make and screen arrays is now much more available than in the past (Gracey, 2007). Studies, to date, have indicated that properly designed and executed gene expression surveys on fish subjected to a relevant change can reveal new insights into physiological responses of stress and welfare patterns, which will be undoubtedly useful for aquaculture.

Acknowledgments

The authors wish to thank the different agencies that supported the stress studies related to this publication: the EU Comission (Imaquanim project), the Spanish Government (Consolider-Aquagenomics project and BFU2009-07354), and the Generalitat de Catalunya (SGR2009-554).

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Chapter 8
Functional Genomic Analysis of Sex Determination and Differentiation in Teleost Fish

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Abstract: In many aquacultured fish species, the production of monosex stocks is highly desirable because usually one sex grows more than the other or produces valuable gametes (e.g., caviar). The sex ratio of a population depends largely on the combined result of the processes of sex determination and differentiation. Fish exhibit a great diversity of sex-determining (SD) mechanisms, and sex differentiation involves the expression of a considerable number of genes in a spatial and temporal order. For genetic selection programs and monosex production in aquaculture, knowing these complex processes is essential. Functional genomics involving the application of microarrays, next-generation sequencing, and other functional genomic approaches, as well as epigenetics, can play an increasingly relevant role in deciphering relevant genes and signaling pathways and how these can be influenced by the environment. Here, we present this chapter on sex determination and differentiation in fish and review the advances made to date using functional genomic analysis using various experimental approaches. We also illustrate some technical complications involving changes in gene expression and DNA methylation. Finally, we suggest some avenues for further research in both model fish species and fish species facing specific problems within an aquaculture context.

Reproduction-Related Problems in Finfish Aquaculture

The reproduction-related problems in finfish aquaculture vary depending upon the species. The main problems that fish may experience under captive conditions are skewed sex ratios, the existence of sexual growth dimorphism, the lack of sexual maturation or side effects on product quality, especially if it occurs too early, and the absence of final maturation or of gamete release (Table 8.1).

Under culture conditions, some species either do not reproduce or exhibit sex-related problems in reproduction. This is, for example, the case of the Senegalese sole (*Solea senegalensis*), where F1 males, which are normally oligospermic, do not produce or release sperm (Cerdà et al., 2008), a situation that has significantly hampered the aquaculture development of this species. On the other hand, and as it is usual in many fish species (Breder and Rosen, 1966; Parker, 1992), including farmed species, one sex outgrows the other (Figure 8.1). This sexual growth dimorphism can favor males (e.g., tilapias) or, more commonly, females (flatfishes, sea basses, etc.). In some cases, as for example in the turbot (*Scophthalmus maximus*), females can be 50% larger than males (Imslad et al., 1997). In other cases, as in the European sea bass (*Dicentrarchus labrax*), the rearing

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

Table 8.1 Reproduction-related problems in finfish aquaculture.

Stage of the life cycle	Reproductive event	Problems/needs for aquaculture
Embryo–larvae	Sex determination	Skewed sex ratios/sex control
Embryo–larvae–juvenile	Sex differentiation	Skewed sex ratios/sex control
Juvenile–adult transition	Onset of puberty	Absence/induction; presence/inhibition
Pubertal–adult transition	Sexual maturation	Deterioration of flesh quality/sterility
Adults	Maturing animals	Environmental interactions/sterility
Adults	Final maturation	Absence of final maturation/induction
Broodstock	Gamete release	Absence of gametes/induction; presence/cryopreservation

conditions result in highly male-biased stocks (Piferrer et al., 2005). Since males grow less than females, then the exploitation is run at a suboptimal capacity. It is then clear that knowing how sex ratio is established will contribute to the development of methods to achieve monosex populations consisting of only the desired sex. A great deal of research toward the development of such sex control methods has been carried out in fish (Piferrer, 2001). Finally,

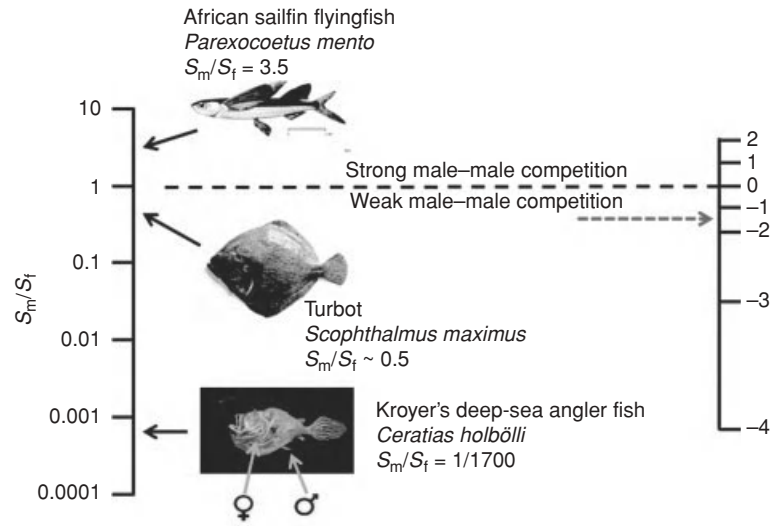

Figure 8.1 Sexual size dimorphism in fish. The vertical ratio scale on the left indicates the relation of the size (mass) of males, S_m, to that of females, S_f. In species with $S_m/S_f > 1$, males are larger than females, while in species with $S_m/S_f < 1$, the opposite is true. The ordinal scale on the right is based on the arbitrary classification of Parker (1992), with the following numerical meanings: 2, males larger than females; 1, males sometimes or slightly larger than females; 0, no size differences; −1, males somewhat smaller than females; −2, males smaller than females; −3, males much smaller than females but not dwarf; −4, true dwarf males. The black dashed line connecting the two scales indicates no size differences, whereas the red dotted arrows represents the mean value of -1.5 ± 0.2 obtained by Parker (1992) on the data of the 118 species present in Breder and Rosen (1966) for which information on sexual size dimorphism was available. Depicted are three examples, showing the extremes and the average situation. In the case of Kroyer's deep-sea angler fish, both sexes are shown (males are very small compared with females, live attached to them, and degenerate most organs but the gonads). See color insert.

many species of fish mature precociously when subjected to the fast growing conditions of modern aquaculture. This affects particularly males, and a well-studied example is the sea bass (Felip et al., 2006). In other cases, such as in the rainbow trout (*Onchorhynchus mykiss*), monosex stocks are desired not only because one sex grows faster than the other, as usually occurs in salmonids, but also because maturation affects the organoleptic properties of the edible parts more in one sex than in the other (Piferrer et al., 2009). Thus, knowledge of the underlying molecular mechanisms present along the brain–hypophisis–gonadal axis would be also helpful for the development of methods to alleviate maturation, including precocious maturation. In addition, in new-farmed species such as the bluefin tuna (*Thynnus thunnus*), many aspects of reproduction are still not known and therefore studies on key aspects of the reproductive axis of this and other potential species for aquaculture are needed (e.g., Aranda et al., 2011).

The physiology, including the reproductive physiology, of an animal can be monitored largely not only by measuring key hormones but also by examining the expression of individual genes or set of genes. Traditionally, this has been achieved through what is known as the "candidate gene approach." Recently, with the advent of genomics, a range of new possibilities have emerged where one can examine the expression of thousands of genes and their interactions all at once, contributing to a better understanding of key signaling and regulatory pathways related to important biological functions such as reproduction. The moment has arrived when this knowledge can be channeled to applied methods within an aquaculture production context.

The knowledge of the sex-determining (SD) mechanism is important for farmed fish species to obtain monosex populations, male or female, according to the species. Sex-associated markers are very useful in this context to identify the genetic sex for precocious sex identification, especially in those species lacking morphological sexual dimorphism. This can aid to know the sex of potential broods in genetic breeding programs to avoid sex bias in the selected population, especially if size dimorphism exists when selection is performed. However, the most relevant application of sex-associated markers is to identify the genetic sex of sex-reversed individuals after hormonal treatment to accelerate the processes for establishing monosex populations (Penman and Piferrer, 2008). This has been usually performed through individual progeny tests involving a lot of space in tanks and time until their maturation and progeny sexing (Piferrer, 2001). The availability of sex-associated markers makes it possible to shorten this process in turbot (Martínez et al., 2009), hirame (*Paralychthys lethostigma*; Patent 93–18132 of South Korea), half-smooth tongue (*Cynoglossus semilaevis*; Chen et al., 2008), Chinook salmon (*Oncorhynchus tshawytscha*), and some other salmonids, where a RT-PCR targeting the growth hormone gene is being used to distinguish males and females (Nagler et al., 2004). In some other species such as the pejerrey (*Odontesthes bonariensis*; Hattori et al., 2009), sturgeon (*Acipenser fulvescens*; Hale et al., 2010), and Pacific halibut (*Hippoglossus stenolepsis*; Galindo et al., 2011), a sex-associated marker has also been identified and could be used for similar purposes. Knowledge of sex determination systems has also been very important to identify highly productive hybrids in tilapia species (Wohlfarth and Wedekind, 1991; Beardmore et al., 2001). Finally, some authors have used sex-associated markers to identify sex reversion as an indication of chemical pollution in some fish species (Flynn et al., 2010).

Introduction to Sex Determination and Differentiation

Sexuality is present in most species in the living world and involves broad and complex machinery for reproduction. This includes

differentiated and specialized cells (sperm and ova), their correspondent organs (testis and ovary) developed from a single undifferentiated primordium, morphological and physiological dimorphism including secondary sexual traits, and a high behavioral diversity to ensure mating, particularly the vast forms of courtship (Williams, 1975). The evolution of such cell, organ, and organismal complexity requires a strong selective pressure on its origins. A general agreement exists on the relevance of sex for generating genetic diversity by enhancing new gene combinations for a fast adaption to new environmental demands (Hines and Culotta, 1998).

Fish, with more than 28,000 species, constitute the largest group of vertebrates, representing more than half of their living species (Nelson, 2006). This group presents all types of reproduction known in vertebrates: gonochorism or separate sexes, by far the most common type, hermaphroditism (<500 species), and unisexuality (Devlin and Nagahama, 2002). Fish live in a large variety of habitats and show highly diverse mechanisms of sex determination (Baroiller et al., 2009a; Desjardins and Fernald, 2009). Sex determination appears essential in this context to ensure a balanced proportion of sexes within populations to make possible their demographic viability. This could explain the high evolutionary plasticity of sex determination in fish, representing a way to adjust the optimum proportion of sexes according to their particular habitat, as demonstrated in some fish species (Conover and Kynard, 1981; Luckenbach et al., 2009). Also, sexual selection could drive changes in the SD system by the existence of antagonistic genes, favorable to one sex but detrimental to the other, that could represent a selective advantage if they were exclusively linked to one sex (Mank, 2009; Mank and Avise, 2009; Charlesworth and Mank, 2010). Accordingly, sex determination systems in fish have frequently demonstrated to be different between species of the same genus (Woram et al., 2003; Cnaani et al., 2008; Roberts et al., 2009; Ross et al., 2009) and even between populations of the same species (Almeida-Toledo and Foresti, 2001).

Sex ratios are an important aspect of populations because they not only determine their reproductive potential but also influence growth dynamics. Therefore, sex ratios are very important in farmed aquatic animals and thus relevant in finfish aquaculture. The sex ratio is the product of the processes of sex determination (Penman and Piferrer, 2008) and sex differentiation (Piferrer and Guiguen, 2008; Figure 8.2A).

Sexual determination in fish involves both genetic and/or environmental mechanisms that establish the gender (male or female) of the organism at conception or early at the initial steps of development, but always before gonad differentiation (Penman and Piferrer, 2008; Valenzuela, 2008). In this sense, sex determination is the event that engages a bipotential gonad to develop as a testis or an ovary (Hayes, 1998). Sex determination in fish can range from genotypic sex determination (GSD) to environmental sex determination (ESD), with temperature-dependent sex determination (TSD) being the most common type of ESD (Figure 8.2B). Most TSD species have no aquaculture or fishery potential, with few exceptions such as the pejerrey. Nevertheless, under some conditions, often including those present in fish farms, many GSD species are capable of responding to the influence of the environment (notably, temperature effects, TE), rendering biased sex ratios. The GSD species in which sex ratios are established by a combination of genetic and environmental influences are referred to GSD + TE species. Examples of GSD species include the salmonids, turbot, and sole, whereas GSD + TE species include the European sea bass and the Southern flounder (*Paralychthys olivaceous*).

In GSD, the establishment of sex is based on the inheritance of major or minor sex factors. The inheritance of sex based on major sex factors, also known as chromosomal sex determination, includes monofactorial and multifactorial SD mechanisms, with the presence of a

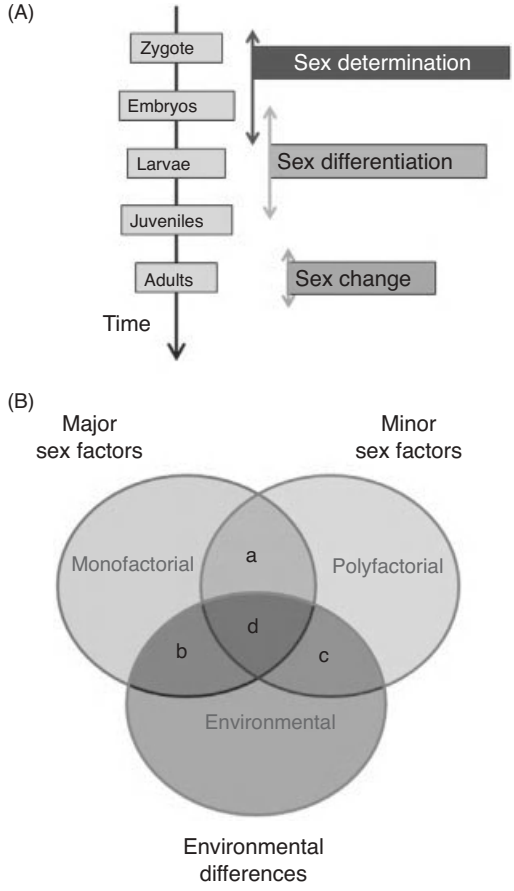

Figure 8.2 Sex determination and differentiation in fish. (A) The processes of sex determination, sex differentiation, and sex change are represented along the timeline of development. The arrows indicate the existence of interspecies differences in the timing of these processes. Sex change only occurs in sequential (also known as consecutive) hermaphrodites. (B) The three major components of fish sex determination. Genotypic sex determination (GSD) driven by major (green circle) and/or minor (blue circle) sex factors, and environmental sex determination (ESD), driven by environmental differences (red circle). Within each circle, the mechanism is indicated. Represented are species with: a, a combination of major and minor sex determining (SD) factors; b, major factors with environmental influences (rare); c, polyfactorial sex determination with environmental influences (common); and d, the theoretically possible combination of the three types of influences. See color insert.

single pair of sex chromosomes or multiple sex chromosomes. Importantly in fish, both male (as in mammals) and female (as in birds) heterogamety exists and sex chromosomes can be homomorphic or heteromorphic (see Table 1 of Penman and Piferrer, 2008, for a complete description). Thus, in fish, the SD mechanism are diverse and often different even in close-related species. This great plasticity of the SD mechanisms is linked to the great species diversity in fish and even sex determination itself may favor speciation (Qvarnström and Bailey, 2009; Roberts et al., 2009). In this context, it is not surprising that the identification of a master SD gene in fish like the *dmy*, also known as *dmrt1(Y)*, gene of the medaka *Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002), is limited to a few species of the same genus (see later).

Traditionally, it has been agreed that the final sex of an individual (phenotypic sex) depends on two sequential processes: the sex determination system of the species and the gonad differentiation process (Valenzuela, 2008). However, recently, these two seemingly distinct processes are viewed as part of a general process leading to gonad formation and sex ratios (Sarre et al., 2004; Quinn et al., 2011; Uller and Helanterä, 2011). Since the discovery of the genes with DM domain involved in sex determination in invertebrates (double-sex, *Drosophila*; Mab-3, *Caenorhabditis elgegans*) and vertebrates (*Sry*), a common origin of sex determination mechanisms in animals was suggested (Marín and Baker, 1998; Miller et al., 2003). However, sex determination systems can change very quickly along evolution (Marín and Baker, 1998). This dichotomy (conservation vs. change) is possible because genetic changes are mostly related to the initial steps of the gene network responsible for gonad development, while most downstream genes across the network are highly conserved (Piferrer and Guiguen, 2008; Charlesworth and Mank, 2010). Although consistent data have been reported and a general agreement exists among researchers on this issue, more

information is required to know the precise relationships and interactions of these genes and their specific weight and balance at the crucial steps of gonad differentiation.

Besides the high evolutionary unstability of the fish SD systems, gonad development, unlike mammals and birds, is also very labile in this group (Devlin and Nagahama, 2002; Nagahama, 2005; Piferrer and Guiguen, 2008). Thus, external factors can influence gonad development, changing the previously determined gonad fate. Likely, both facts are interconnected, and the gonad development flexibility in fish offers more opportunities to change SD systems along evolution. Thus, both genetic and environmental factors are involved in the phenotypic sex in fish, and this influence can occur both at the initial stages of development related with sex determination, or later during gonad differentiation. Accordingly, GSD and ESD may represent the extremes of a continuum, with variable weight of both factors between them (Barske and Capel, 2008; Penman and Piferrer, 2008). However, information is lacking to understand the actual importance of environmental factors on sex determination.

Genotypic Sex Determination

Evidences of Genetic Factors Underlying Sex Determination in Fish

One of the first clues of the genetic basis of sex determination in fish was the identification of sex-associated heteromorphisms at specific chromosomes, the sexual pair, thus differentiated from the other members of the karyotype, the autosomes (Bull, 1983). However, karyotypic studies related to sex determination have demonstrated that, unlike mammals and birds, most fish species do not have differentiated sex chromosomes. The male heterogamety (XY) is the most common reported system, but many species have female heterogamety (ZW), and more occasionally, multiple chromosome systems (Almeida-Toledo and Foresti, 2001; Devlin and Nagahama, 2002; Penman and Piferrer, 2008). Given the low resolution of optical microscopy to differentiate sex chromosomes in fish, researchers have looked for an alternative in the tenfold longer meiotic chromosomes to detect mispairing tracts at the synaptonemal complex as an indication of the sex differentiated region with variable success. The lack of differentiation at the sexual pair has led many researchers to suggest that sex chromosomes in fish are at a primitive stage of evolution, and therefore they are valuable for investigation of sex determination mechanisms (Peichel et al., 2004; Kikuchi et al., 2007). While it can be accepted that fish chromosomes are at an early stage of evolution (compared with those of, say, mammals), this view does not take into consideration the importance of sex determination for adaption or as a mechanism of speciation within fish (Roberts et al., 2009). Thus, the great evolutionary turnover of the SD system, which hampers the accumulation of repetitive DNA in the SD gene-bearing chromosome (Y/W), is related to the low differentiation of the sex chromosome pair in fish.

Other indirect evidence of the genetic basis of sex determination was obtained from the sex ratio of progenies of chromosome set manipulated individuals, such as gynogenetics and triploids, or from steroid-treated sex reversal individuals (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). The exclusive female constitution of gynogenetic genomes provides information on the SD system, especially in a XX/XY system, where all female progenies are expected. If ZZ/ZW is the underlying system, male offspring always will be present, but the interpretation is more complex and will depend on the distance of the SD region to centromere and on the viability of WW offspring (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). Induced triploids, on the other hand, are constituted by the combination of two female and one male genomes (Piferrer et al., 2009), and therefore, a female bias should be expected in triploid progenies following quantitative

criteria. However, this bias, if it exists, will depend on the same factors outlined before and, additionally, on the dominance of the Y or W chromosomes over their correspondent X and Z chromosomes. Deleterious alleles associated to the Y or W chromosomes can further complicate the situation, deviating sex ratio from the expected proportions. An example of this was reported in turbot, where the presence of all-female offspring first suggested the presence of a XX/XY system (Piferrer et al., 2004). However, further association analysis using a genetic map and analysis of progenies from sex reversal individuals supports the existence of a ZZ/ZW system (Haffray et al., 2009; Martínez et al., 2009).

Progenies from sex reversal individuals may also provide key information on the SD mechanism. Thus, all-female offspring should be expected in a XX/XY system when genotypic female larvae treated with androgens such as 17α-methyltestosterone or 17α-methyldihydrotestosterone are used as fathers (neomales), while all-male progenies should be obtained in a ZZ/ZW system when genotypic male larvae treated with estradiol-17ß (E_2) are used as mothers (neofemales). Data from gynogenetics, triploids, or sex-reversed progenies would be additionally complicated if more than one genetic factor or environmental variables are involved in sex determination. Therefore, these approaches work well when the SD mechanism is monofactorial. Finally, in some species, sex-linked morphological or biochemical markers have demonstrated the underlying genetic basis of sex determination (Allendorf et al., 1994; Matsuda, 2005; Roberts et al., 2009; Tripathi et al., 2009).

Sex Determining Genes

Contrary to the apparent conservation of gene differentiation network in vertebrates, a great diversity in the gene-switching mechanisms has been suggested (Marín and Baker, 1998; Barske and Capel, 2008; Graves and Peichel, 2010). Because of the knowledge of SD systems in *Drosophila melanogaster*, mammals, and birds, it was easy to assume that a single switching gene was the basis of the subsequent gonad differentiation in fish. In mammals, the expression of the *SrY* gene, only present in the male genome, determines the differentiation of the somatic Sertoli cells, which, in turn, induces the male pathway in the primordium through activation of *Sox9* (Lovell-Badge and Hacker, 1995; Kim and Capel, 2006). An important fact observed during mammalian gonad differentiation is the existence of interaction between antagonic genes, which determine the male or female pathways at the beginning of development. Thus, *Wnt4* and β-*catenin,* key genes for ovary development, are inactivated by the action of *Sox9* and *Fgf9*, which determine testis development. If *Sox9* is not activated by *SrY*, as occurs in females, *Wnt4* and β-*catenin* drive development toward an ovary (Nef and Vassalli, 2009). This antagonic interaction could occur at other key points across gonad development, especially in fish, where it is possible to revert gonad development by hormone induction or environmental factors, and may explain the variability of the SD system in this group and their plasticity in evolutionary terms. This suggests gene networks rather than gene pathways or cascades, as usually surmised, because they suggest a linear concatenated developmental event.

Although the SD region has been identified in several fish species like medaka, stickleback (*Gasterosteus acculeatus*), platyfish (*Xiphophorus maculatus*), Takifugu (*Takifugu rubripes*), rainbow trout, catfish (*Clarias gariepinus*), turbot, pejerrey, Nile tilapia (*Oreochromis niloticus*), Japanese flounder (*Paralichthys olivaceus*), half-smooth tongue sole (*Cynoglossus semilaevis*) and Pacific Halibut (*Hippoglossus stenolepis*), the responsible gene has only been elucidated in one species, the medaka (Matsuda et al., 2002; Nanda et al., 2002). In the medaka, the SD gene is named *dmy* or *dmrt1b* and is a copy of *dmrt1* that has taken the role as SD gene.

Sex Determining Mechanisms in the Most Studied Fish Species

Medaka

Medaka*, O. latipes*, with a XX/XY SD system, is the only fish species where the SD gene has been identified. *dmy* (*dmrt1bY*) is the SD master gene and was originated by duplication of *dmrt1*, involved in testis development in vertebrates, between 4 and 10 million years ago (Mya) (Matsuda et al., 2002; Nanda et al., 2002; Matsuda, 2005). In addition, to associate dmy with sex at the species level, functional experiments, like frameshift and low expression mutants, demonstrated a full or partial bias toward females, confirming its crucial role for male fate. Also, transgenic *dmy* XX individuals developed as fully functional males. The same SD gene has been reported in the two closely related species *O. curvinotus* and *O. luzonenesis*, but it was not the SD gene in the congeneric species *O. mekongensis* (Kondo et al., 2003), and in some species of the genus *Oryzias,* a ZZ/ZW system has been described. At least two different chromosome pairs are involved in the origin of *dmy* in *Oryzias* (Tanaka et al., 2007). This reflects well the evolution pattern of SD genes in fish and has been described in many other genera. Medaka is one of the fish species where the SD region has been studied with more detail. The SD region of this species shows reduced recombination and an enrichment in repetitive sequences and pseudogenes, as expected according to theory. *dmy* is expressed in the Sertoli somatic cells in a continuous way from embryo to adult, but exclusively in the testis (Matsuda, 2005).

Tilapia

Tilapias are a diverse group of fish comprising more than 70 species of Cichlids that originated 5 Mya. Two main SD chromosomes, corresponding to linkage group 1 (LG1) and LG3, have demonstrated to be associated with sex determination in different species, and other minor factors have been reported in studies with hybrids by QTL analysis (Shirak et al., 2006). Molecular studies on these chromosomes demonstrated the suppression of recombination, the accumulation of transposons, and the presence of lethals in the heterogametic sex. Some candidate genes have been discarded through mapping studies (*cypb1a*), while others have been postulated as SD genes (*amh* and *dmrt2*). According to Cnaani et al. (2008), in six tilapiine species, the SD locus in LG1 is a dominant-male determinant and defines a XX/XY system (*O. niloticus* and *Tilapia zilii*), while LG3 is a female-dominant ZZ/ZW system (*O. karongae* and *T. mariae*). However, both systems are active in *O. aureus* and *O. mossambicus* (Lee et al., 2004). All-male offspring hybrids have been obtained when crossing XX females and ZZ males (Wohlfarth and Wedekind, 1991). The evolution of sex determination in tilapias suggests a minimum of two transitions between XY and ZW, with an intermediary state with both systems active (ZX and XY). The LG3 locus would be the ancestral state according to its higher differentiated state with accumulation of SINEs and LINEs and the evidence of a meiotic mispairing (Carrasco et al., 1999). In spite of strong genetic effects, TE have also been described in tilapias (Baroiller et al., 2009b), but whether these represent an ecologically relevant alteration of the SD system or only occur under very exceptional environmental or laboratory conditions needs to be clarified (Ospina-Álvarez and Piferrer, 2008).

Poecilids

The platyfish has a multifactorial SD system with three sex chromosomes (X, Y, W), males being XY or YY, and females XX, XW, or WY at the population level. However, at the individual level, there is only one single pair of sex chromosomes. No evident heteromorphism could be detected between sex chromosomes based on pairing anomalies (Traut and Winking, 2001). Sex chromosome differences

have been found only in one population (Nanda et al., 1992), whereas accumulation of repetitive sequences was observed in the Y chromosome (Nanda et al., 2000). The main SD region is flanked by two receptors of tyrosin kinase gene and includes genes related to pigmentation and sexual maturation (Schultheis et al., 2006). As in medaka, a large amount of transposon elements have been detected associated to the SD chromosome pair (Froschauer et al., 2002).

The guppy (*Poecilia reticulata*) is another poecilid with a XX/XY system and coloration-related genes are associated to the Y chromosome. This chromosome has no homology to the LG12 of medaka by comparative mapping, strongly suggesting an independent evolution of its SD gene. As in medaka, recombination frequency differences have been detected between males and females in two regions adjacent to the pseudoautosomal segment, one of them not directly related with sex determination, which represents an intriguing observation (Tripathi et al., 2009).

Stickleback

The three-spine stickleback is the main model fish species for adaptive evolutionary studies (Peichel et al., 2004). Mapping association studies enabled the location of the SD region in the distal section of LG19. This region does not appear to be heteromorphic with microscopy resolution, but the fixation of heterozygotes for multiple loci in males suggests a XY mechanism. Additionally, recombination is reduced in males in this region, although not in the whole genome. The analysis of BAC clones in a 250 kb region provided strong evidence for substantial differential sequence composition between males and females (only 64% homology and 87 kb longer in males), denoting the accumulation of repetitive sequences and duplications (Peichel et al., 2004). A notable variation has been observed in the SD system in different stickleback species, which were originated around 10 Mya. Thus, the nine-spine stickleback (*Pungitius pungitius*) also showed XY mechanism associated with LG12 using molecular markers, while *Gasterosteus weathlandi* presented a multiple chromosome system because of the fusion of LG12 and LG19, the only fusion case reported to date between two extant SD chromosomes. Finally, the variable SD landscape within stickleback group is supported by the presence of a ZZ/ZW system in four-spine stickleback, *Apeltes quadracus,* and brook stickleback, *Culaea inconstans*. Hybridization with LG12 and LG19 probes demonstrated that the ZW pair in these species is not homologous to those previously described in other stickleback species (Ross et al., 2009; Shapiro et al., 2009).

Evolution of Sex Chromosomes

Suppression of recombination is common in the evolution of sex chromosomes to avoid the breakage of linkage between the SD and the sex-associated benefit alleles (Charlesworth and Charlesworth, 1997). This phenomenon has also been detected in the incipient SD regions of many fish species (Schartl, 2004). However, excluding some cases, recombination suppression occurs in a small genomic tract where these genes are located, and it does not extend over most of the sex chromosome pair, as occurs in mammals and birds (Bergero and Charlesworth, 2009). It is not clear if this suppression occurs by the presence of inversions or as a modulation of the recombination mechanism itself, but both could be involved (Bergero and Charlesworth, 2009). Evidence of recombination in the SD region in sex reversal individuals supports the second hypothesis. However, the existence of a homologous region is still necessary for a correct segregation of both members of SD pair during cell divisions (Karhunen, 2011). Lack of recombination is always associated with heterogametic sex, while in species with heteroquiasmia (different number of quiasmae between sexes), females show more frequently higher recombination rates (Hedrick, 2007). These differences

seem to be more associated with gametic selection than with the type of SD system (Lenormand and Dutheil, 2005).

Another recurrent event during SD pair evolution is the accumulation of repetitive DNA, repetitions and deleterious alleles in the SD gene-bearing chromosome (W or Y; Schartl, 2004; Volff et al., 2007). This would lead to a progressive inactivation and heterochromatinization of these chromosomes in successive drift events driven by Muller's ratchet according to theoretical predictions (Charlesworth and Charlesworth, 1997). This occurs because most mutants are recessive and the heteromorphic chromosome is always in heterozygous condition, so preventing their selection. This pattern has been observed in most species where the SD region has been analyzed (Nanda et al., 1992; Peichel et al., 2004; Nagahama, 2005). However, most fish species show an incipient degree of differentiation and there are exceptional cases where a strong heteromorphisms exist in the sexual pair. In fact, most WW and YY genotypes in fish seem to be viable (Penman and Piferrer, 2008). Also, lack of dose compensation mechanisms have been observed in fish (Devlin and Nagahama, 2002; Volff et al., 2007). This may be related to the evolutionary instability of SD systems in fish, but an alternative or complementary explanation suggests that sex reversal is a sort of a youth fountain for sex chromosomes (Perrin, 2009; Karhunen, 2011). This hypothesis relies on the developmental lability of SD in fish and thus the frequent sex reversal at particular individuals by environmental factors. In these cases, since recombination pattern appears to be associated with sex, crossing over could occur at the SD region, breaking the isolation of this gene block and facilitating the homogenization with its Z or X counterparts (Charlesworth and Mank, 2010). Sexual reversion can be favorable in this context under specific selection models as demonstrated by Karhunen (2011).

A derived condition of the SD chromosome evolution is that genetic diversity should be lower at the SD gene-bearing pair. This is because of its lower effective population size, therefore been subjected more intensively to drift phenomena, but also by the lower variability associated with new favorable selective regions (Ellegren, 2009). This situation has been reported in turbot, where microsatellites associated with the SD region showed lower variation than those in the whole genome (Martínez et al., 2009). More precise evaluations of genetic diversity associated to the SD region will be possible with next-generation sequencing (NGS) technologies by using restriction-site associated DNA (RAD) or genotyping by sequencing (GBS) approaches (Baird et al., 2008; Elshire et al., 2011).

Environmental Sex Determination

Different studies have emphasized the relevance of environmental factors in sex determination (Valenzuela et al., 2003; Valenzuela, 2008; Baroiller et al., 2009a). TSD is well established in reptiles. However, in fish, the situation is more complex. Although TSD has been repeatedly assumed solely based on the evidence of sex ratio response to temperature in up to 61 species (e.g., Baroiller et al., 2009a), a more critical assessment showed that in many cases, these largely laboratory-based observations did not reflect the actual situation in nature. Thus, TSD is indeed present in fish, also reflecting the extraordinary plasticity in sex determination and differentiation, but its abundance can be unambiguously attributed to less species than originally assumed (see Ospina-Álvarez and Piferrer, 2008, for review).

However, there are GSD or TSD species, and in fact in many cases, sex determination depends on the combination of both genetic and environmental factors. An important aspect to be taken into account is that regardless of whether one species has TSD or GSD, under certain circumstances (e.g., under extraordinary natural conditions or when pushed in the laboratory), fish can respond to temperature in terms of resulting sex ratios, and such effects of

temperature on sex ratios apparently involve the same mechanism as TSD. Differences of temperature can influence critical biochemical routes involved in sex determination, especially in poikilothermic organisms (Devlin and Nagahama, 2002). It has been suggested that high temperature can inhibit the action of gonadal aromatase, likely through its transcriptional activators *foxl2* and *ftr1*, but it is also possible that heat-shock proteins will be involved in this process (Luckenbach et al., 2009). Because of its central role in sex differentiation in all nonmammalian vertebrates (Guiguen et al., 2010), aromatase is likely a candidate to be involved in the regulation of sex ratios by temperature. However, despite many research efforts, a plausible mechanism has not been elucidated. Recently, Navarro-Martín et al. (2011) have shown in the European sea bass that males have double the amount of DNA methylation in the promoter of gonadal aromatase than females. Further, high temperature during early development induces hypermethylation of this promoter in females, approaching the values of males, with a concomitant reduction in aromatase expression and the corresponding increase in the number of males. This is then the first time that an epigenetic mechanism is involved in TSD in any animal. Differences between GSD and TSD begin at conception where the genetic sex is specified according to its genetic constitution, while the thermosensitive period occurs later, although at the beginning of gonad development (Valenzuela and Lance, 2004). The pivotal temperature (Tp) has been defined as one that determines a 1:1 ratio, while other temperatures, higher or lower, produce a deviation either towards a higher proportion of males or females (Barske and Capel, 2008). In reptiles with TSD, three patterns of sex ratio exist in response to temperatures (Valenzuela and Lance, 2004). In fish, a similar situation was accepted, also with three sex ratio patterns in response to temperature. However, a critical assessment of the available data, where the incidence of TSD in many previous cases was dismissed, revealed that in fish species with sex ratio in response to temperature, there is only one pattern, i.e., an increase of males with increasing temperatures (Ospina-Álvarez and Piferrer, 2008).

Regarding the possible role of nongonadal tissues on sex determination–differentiation, it has been suggested that the brain, which develops before gonad in embryos, could have an influence by transferring an environmental signal for defining the fate of the undifferentiated gonad. This is conceivable in hermaphrodites species, where social cues often determine when sex change occurs. Thus, brain aromatase (*cy19a1b*) could play a role for signal transduction, as suggested by the female-associated expression in brain of the orange-spotted grouper, *Epinephelus coioides* (Nagarajan et al., 2011). However, despite some claims (see Blázquez and Somoza, 2010, for review) in gonochoristic species, the evidence so far does not support this hypothesis, something that does not prevent the existence of not noncausal associations between events in the brain and in the gonads. Thus, in pejerrey, the preoptic neurons have been reported to be correlated with sex determination at different temperatures, which suggests a way for signal transferring through hypothalamus–pituitary axis. In the sea bass, sex-related changes in FSH expression have been correlated with sex differentiation (Molés et al., 2011). TE could be rather related to particular embryo traits than with sex determination itself, and therefore be an indirect determinant of gonad fate. Thus, growth rate, which is strongly influenced by temperature, could be responsible for the gonad determination (Baroiller et al., 2009a). In southern flounder, higher temperatures determine the maximum growth rate and the highest proportion of females (Luckenbach et al., 2009). Compensatory growth observed in some species could influence female differentiation after maintenance at low temperatures, as suggested by Baroiller et al. (2009a). It should be noted that females are usually, but not always, the larger sex in fish (Parker, 1992) and a relationship between sex determination and growth has been

detected in several species (Cnaani et al., 2004; Loukovitis et al., 2011). Additionally, the number of cells of the ovarian primordium is higher than those of the testis, and female primordium begins differentiation before the male one (Lewis et al., 2008). According to this, a role for insulin growth factor-1 has been suggested at the beginning of primordium differentiation (Schlueter et al., 2007; Luckenbach et al., 2009).

Not many cases have been reported in the wild where clear-cut evidences of temperature influence have been demonstrated. The Atlantic silverside, *Menidia menidia*, an atherinid fish, is likely the only species where this influence has been demonstrated with confidence in wild conditions. In this species, a temperature cline has been related to sex ratio, as a way to respond to sex-specific selective pressures. At lower temperatures, the number of females increases in the populations in response to their longer period of gonad development. Luckenbach et al. (2009) also observed Tp changes with latitude in flatfish species, suggesting selective pressures to adjust sex ratio. In the same way, the low sensitivity temperature observed in barfin flounder could indicate an adaption to the low water temperatures of its natural environment, and in fact, all-male offspring have been obtained at high temperatures. Other examples of increasing proportion of males by temperature are pejerrey, tilapia, medaka, and marbled sole (*Pseudopleuronecthes yokohamae*). Conversely, no effects of temperature on sex determination have been detected in the mummichog (*Fundulus heteroclitus*), mosquitofish (*Gambusia affinis*), bloater (*Coregonus hoyi*), sheepshead minnow (*Cyprinodon variegatus*), and Atlantic halibut (Luckenbach et al., 2009).

More studies will be necessary in the future to elucidate the influence of temperature on sex ratio in natural conditions (Ospina-Álvarez and Piferrer, 2008), although this is not an easy task (Luckenbach et al., 2009). The role of temperature as the only SD factor in some species should be verified with data that are more consistent. It is possible that temperature is only modulating gonad development and adapting it to particular environmental conditions under selective pressures. Even in the case of the Atlantic silverside, where ecological and evolutionary considerations have been taken into account, some populations have demonstrated a strong GDS (Yamahira and Conover, 2003). In addition, it should be noted that the effect of temperature on sex determination has a genetic basis itself and an interaction between families and temperature effect has been reported in several species (Schultz, 1993; Vandeputte et al., 2007). Finally, other environmental effects such as pH, hypoxia, and social factors have claimed to be involved on sex determination (reviewed by Guerrero-Estévez and Moreno-Mendoza, 2010). All the information obtained to date on SD in fish suggests that the sex ratio response to temperature may be treated as a quantitative threshold trait as suggested by some authors (Mittwoch, 2006).

Evolution of Sex Determining Systems

The constant duality of sex all over the history of life (males and females) could suggest a high conservation of the molecular mechanisms switching and driving the development of the undifferentiated primordium toward the definitive sex of the individual. However, within vertebrates, while mammals and birds show a fairly high conserved mechanism of sex determination, in contrast, fish, amphibians, and most reptiles show a great diversity involving both genetic and environmental factors (Valenzuela, 2008). Even within mammals, where *SrY* is the gene responsible for testis determination, monotremata show a different multichromosomal sex determination mechanism (involving 5X + 5Y chromosomes), and recently, three species in the rodent line have demonstrated a different switching gene to *SrY* (Graves and Peichel, 2010). The high conservation of sex determination within birds and mammals has probably to do with their high development

homeostasis, including constant body temperature (Barske and Capel, 2008).

Phylogenetic analysis does not support a monophyletic origin for the different SD mechanisms in gonochoristic fish, since their high heterogeneity and mosaicism in most fish groups (Devlin and Nagahama, 2002). In addition, TSD systems seem to have evolved several times (Ospina-Álvarez and Piferrer, 2008) as a mechanism, which permits to adjust the optimal sex ratio according to environmental cues. On the other hand, hermaphroditism and unisexuality are also of polyphyletic and ephemeral origin, the last one always being preceded by species hybridization events. The lack of homology between the different SD chromosomes in fish suggests an independent evolutionary origin from different autosomes (Mank et al., 2006). The high development gonad plasticity of fish associated with selective pressures derived from social status, ecological conditions, and population composition is likely based on the evolutionary plasticity of sex determination in this group (Mank, 2009). As outlined before, a gene network with balanced mechanisms along a hierarchical development pathway could be responsible for this flexibility, and evolutionary models have been developed to explain transitions between different SD mechanisms (Erwin and Davidson, 2009). However, most genes in this network seem to be conserved and only changes have been suggested to occur at the top of the hierarchy (Marín and Baker, 1998; Barske and Capel, 2008; Graves and Peichel, 2010). Genome duplications during the course of vertebrate evolution, and particularly within teleosts, would offer new opportunities for the appearance of new SD genes (Mank et al., 2006). Recycling the same genes as SD masters in different unrelated groups could indicate their better properties to cover this function, as also occurs with recycling of the same chromosomes in different lineages in teleosts (Graves and Peichel, 2010).

Sexually antagonistic coevolution has been suggested as one of the main selective pressures for the evolution of SD master genes. This can arise from different sex-associated traits like courtship, mating, and parental investment, when the optimum phenotype differs between sexes (Chapman, 2006). In addition, the existence of notable differences on gene expression between sexes could provide many opportunities for the emergence of antagonistic genes (Mank, 2009; Charlesworth and Mank, 2010). If a favorable allele for one sex is associated with a new autosomic determinant, a new mechanism can emerge. This can be polymorphic under certain conditions, but can definitively be shifted to a new SD system (Ezaz et al., 2006; van Doorn and Kirkpatrick, 2010). Models have been developed to study the evolution of a new autosomic mutation in a sexual conflict scenario and the likelihood to replace the previous SD system (MacCarthy et al., 2010; van Doorn and Kirkpatrick, 2010). The existence of fish taxa with different SD systems (ZW and XY) either in separate species or coexisting in a single species, such as in some tilapia and stickleback lineages, suggests that this indeed does occur in the wild. Different sexual antagonistic genes have been reported to date, some of them related to favorable coloration and sex-associated patterns. Thus, in guppies and Cichlids, coloration genes have been detected to be associated with the SD region, determining the expression of particular alleles only in one sex (Schartl, 2004; Volff et al., 2007; van Doorn and Kirkpatrick, 2010). In Cichlids of the Lake Malawi, the orange-blotch coloration, which is associated with the SD W region, facilitates the camouflage of females. This pattern breaks the mating coloration of males. This new SD locus would be dominant over the previous XX/XY one existing in LG7 in most Cichlids (Roberts et al., 2009). The new SD loci could even arise on sex-associated B chromosomes, usually considered as selfish DNA (Camacho et al., 2000) since different evidences of sex-associated B chromosomes have been reported in fish (Yoshida et al., 2011).

Some authors have suggested that changes in the SD mechanism could drive speciation (Qvarnström and Bailey, 2009; Ser et al., 2010).

In Cichlids, the 19 studied species showed XY (LG7) or ZW (LG5) mechanisms, the ZW system being epistatic over the XY in hybrids. Evidence suggests that other determinants are present in other LGs, which can be acquired in a new sexual antagonistic scenario. It is important to note that LG5 and LG7 are not homologous to LG1 and LG3 from tilapiine species, as revealed by comparative genomics studies. A postzygotic isolation model could emerge in this scenario if hybrids showed lower fitness than species showing opposite SD mechanisms. These data support the idea of sex determination as a quantitative threshold trait (Mittwoch, 2006).

Sexual ratio has also been invoked as an important factor driving SD mechanism, since this is an essential demographic parameter for the viability of populations (Penman and Piferrer, 2008). Selective pressures for the maintenance of optimum sex ratio has been claimed to explain the rapid transitions between alternative SD systems (Quinn et al., 2011). However, some authors have suggested that sexual ratio cannot be the sole force driving this process and in fact more than one SD locus can remain polymorphic at equilibrium, as demonstrated in domestic fly, which possesses a polygenic SD mechanism (Kocher, 2004).

Sex Differentiation

The undifferentiated primordium is bipotential and therefore capable to develop a male or a female gonad through alternative development and differentiation pathways. In contrast, with sex determination, the genes and networks implicated in fish sex differentiation appear to be quite conserved throughout the species and even across the different groups of vertebrates (Piferrer, 2011). As in the rest of vertebrates, fish gonads have two major functions: gametogenesis and steroidogenesis. As a gland, the major products of synthesis of the gonads are the sex steroids, androgens (mainly testosterone, 11ß-hydroxyandrostenedione, and 11-ketotesttosterone), and estrogens (mainly E_2). Androgens and estrogens are implicated in male and female sex differentiation, respectively. The gonads also produce progestagens, but there is no evidence of their implication in sexual differentiation. Thus, the major products of synthesis of the adult gonads, sex steroids, are also responsible for their differentiation. Therefore, it is not surprising that the genes implicated in the formation and the differentiation of the gonads can be grouped into three broad classes: those that are implicated in organogenesis (and thus ubiquitous in the formation and differentiation of many organs of the body), those implicated in male differentiation, and those implicated in female differentiation. Most of these genes fall in one of the following categories: steroidogenic enzymes, sex steroid receptors, transcription factors, and growth factors. A description of these genes types related to sex differentiation can be found in Piferrer and Guiguen (2008). Among these genes, the steroidogenic enzyme aromatase (*cyp19a*) occupies a prominent place. This enzyme is solely responsible for the irreversible conversion of androgens into estrogens and thus determines the balance between these two antagonist sex steroid types. Currently, it is thought that in most nonmammalian vertebrates, female sex differentiation depends essentially on the stimulation of *cyp19a* through a positive feedback loop, while male sex differentiation would follow after inhibition of *cyp19a* expression (Guiguen et al., 2010). This inhibition of *cyp19a* would be achieved by different mechanisms, such as high temperature in a TSD or GSD+TE species, a male master SD gene in a GSD species with a monofactorial system of sex determination, or by the additive effects of several male-promoting genes in a polyfactorial system.

The conserved role of aromatase in female sex differentiation in fish (reviewed in Guiguen et al., 2010) is exemplified by the fact that the effects of temperature on sex ratios are invariably linked to an inhibition of *cyp19a* expression. This has been observed in species with a strong

genetic basis of sex determination (pure GSD species), such as the Atlantic halibut (van Nes and Andersen, 2006); in species with a combination of GSD and ESD (GSD + TE species), such as the olive flounder (Kitano et al., 1999); or in species with TSD, such as the pejerrey (Karube et al., 2007).

Homologous genes for most factors involved in mammalian development have been found in fish, suggesting a high conservation of the gonad differentiation network in vertebrates. In this context, it has been demonstrated the relevance of *Sox9* at the initial steps of testis differentiation in all vertebrates including fish (Graves and Peichel, 2010; Guerrero-Estévez and Moreno-Mendoza, 2010); the importance of *dmrt1* in testis differentiation just after the action of the SD gene (Nagahama, 2005; Hong et al., 2007); the expression of *amh* in the Sertoli cells and in adult ovaries in granulosa cells surrounding the oocytes (Klüver et al., 2007); that *Dax1* downregulates *cyp19a* expression in ovarian follicles (Nakamoto et al., 2007); that *foxl2* plays a decisive role in the ovarian differentiation by regulating *cyp19a* expression (Wang et al., 2007); the expression of *Vasa* and SSB-4, gene markers for gonad differentiation in primordial cells (Li et al., 2009); the relevant role of estrogens and steroidogenic enzymes for gonad differentiation (Matsuda, 2005); and the key role of *cyp19a* in the balance between androgens and estrogens (Nagahama, 2005).

Approaches to Study Genomics of Fish Sex Determination and Differentiation

Although relevant information has been obtained to understand sexual determination and its evolution in fish, more data are needed to fulfill important gaps and to solve current controversies. In this sense, the vast amount of data coming from genomic technologies will be decisive. NGS technologies have greatly reduced their costs until 1–10$/Mb (Nature Editorial, 2010), making possible the comparison of male and female whole genomes at a reasonable cost. Enrichment or chromosome microdissection of specific sex-associated genomic regions may enable to focus the analysis on a small genome fraction for a higher efficiency (Subramanian et al., 2005; Poonlaphdecha et al., 2008). Also, the new methods combining the use of restriction enzymes (RE) with NGS (RAD; GBS) make it possible the comparison of a vast amount of genetic markers randomly chosen across the genome to look for associations at specific characters, including sex determination (Baird et al., 2008; Elshire et al., 2011). The application of accurate gene expression techniques, including the most resolving oligo-microarrays (Canales et al., 2006; Patterson et al., 2006) and their extension to subgenomic regions related to regulation (Swami, 2009; Licatalosi and Darnell, 2010), will allow obtaining detailed maps of expression and key regulation genes along development in parallel with cytological events (Baron et al., 2005). Finally, comparative genomics tools will be valuable for obtaining information on candidate SD genes from model fish species, once identified the master region through association studies (Paterson, 2006; Pennisi, 2007).

Genomic Approaches for the Study of Sex Determination

Genetic markers covering the whole genome with variable density have been used to look for associations with sex or to detect sex-specific genomic regions, to understand the SD genetic mechanism. These strategies rely on the existence of a large sex-differentiated genomic region and/or on the existence of strong genetic effects associated to one or a few genomic regions. Thus, the success to find the SD region depends on the SD mechanism, the genomic properties of the SD region itself, and the choice of an appropriate searching approach. Identification of sex-specific genomic

regions has been carried out by genomic screening with random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) techniques, and using subtractive hybridization genomic libraries. This approach has demonstrated severe limitations since large differentiated genomic regions are not common within fish and success is highly dependent on the effort applied. Sex-associated AFLPs have been successfully identified in the half-smooth tongue sole, with one of them producing a useful sequence characteristic amplified region (SCAR) with female-specific amplification (Chen et al., 2007); in the rainbow trout (Felip et al., 2005); in three-spine stickleback (Griffiths et al., 2000); and in Nile tilapia (Ezaz et al., 2004). In addition, two sex-associated RAPDs were detected in catfish (Kovacs et al., 2000) and four in turbot (Casas et al., 2011).

Association studies have been usually successful since strong genetic effects commonly underlie sex determination. Their success depends on the quality and density of the genetic map applied and the use of appropriate family crosses. They can be applied using more sophisticated QTL analysis (Cnaani et al., 2004; Martínez et al., 2009; Shapiro et al., 2009) or by simple statistical association tests (Peichel et al., 2004; Kikuchi et al., 2007; Koshimizu et al., 2010). An interesting shortcut to this approach is represented by the use of pools of male and female DNA following the so-termed bulk segregation analysis (BSA) (Felip et al., 2005; Casas et al., 2011). Very recently, the application of NGS combined with RE genome fragmentation enables to obtain a very large panel of SNPs covering the whole genome, thus ensuring a powerful association screening. RAD analysis is based on the digestion of the genome with an appropriate RE, followed by sequential ligation of two adaptors types, and finally, by a massive sequencing with Illumina or SOLiD sequencing technologies (Baird et al., 2008). GBS method uses a similar approach, with a simpler library construction protocol, but additionally, permits discarding most of the highly repetitive genomic fraction present at some genomes, which could interfere with the analysis (Elshire et al., 2011). Both approaches, because of the depth of the run, provide a large amount of sequences for a common genomic subsample of the analyzed individuals, allowing the detection of thousands of SNPs covering the genome. Since barcoding (a small nucleotide tract included in the adaptor) can be used, one can sequence different phenotypes in the same run (for example pools of males and females) using different codes and subsequently look for SNP sex-associated genetic markers. Although very recent, this strategy promises to be highly effective and much more cost-efficient to detect sex-associated genetic markers.

Once a candidate genomic region has been identified, the challenge is to locate the SD gene, a difficult task considering that a single SD gene has been identified so far in fish, a duplicated copy of *dmrt1* named DMY in the medaka, *O latipes* (Matsuda et al., 2002; Nanda et al., 2002). A straightforward approach is to map candidate genes, to look for their cosegregation within the main SD genomic region. Thus, in tilapia hybrids, Shirak et al. (2006) identified two candidate genes (*Amh* and *Dmrt2*) related with the sex-differentiation process, which were associated to a QTL in LG23. However, this approach has enabled to discard better specific candidate genes rather than to identify potential SD master genes (Alfaqih et al., 2009; Viñas et al., in press). Cloning and sequencing the SD region can be facilitated by the construction of BAC libraries. Such libraries have been obtained in several fish species (reviewed by Shao et al., 2010) and used for locating the SD master gene and/or characterizing the SD-differentiated region in medaka (Matsuda et al., 2002), platyfish (Froschauer et al., 2002), three-spine stickleback (Peichel et al., 2004), and tongued sole (Shao et al., 2010). Starting from markers adjacent to the SD region, a local physical map can be constructed, assembling individual BACs and subsequently searching for putative genes

associated with the sex differentiation pathway. Although BAC libraries have been considered very useful, the recent advances on NGS technologies permits to obtain the whole genome sequence at low cost, and hence accessing the complete genome sequence of the region of interest. Furthermore, if the SD-bearing chromosome is identifiable in mitotic metaphases or prophasic meiotic chromosomes, the whole chromosome or a segment can be sequenced with much lower cost after microdissection with micro-handling microscopy techniques (Poonlaphdecha et al., 2008). However, the SD gene has not been identified in most model species despite the availability of the sequence of a considerable part of their genome. An advantage of BAC libraries is that they contain the physical DNA, which can be used as a probe in SD hybridization studies. Ross et al. (2009) used sex-associated BAC probes from *Gasterosteus aculeatus* to carry out comparative FISH (fluorescence in situ hybridization) experiments on several stickleback species demonstrating common or different origins of the SD region in species of this group. Phillips et al. (2001) used painted probes from the SD chromosome regions of *Salvelinus* and *Oncorhynchus* to compare the origin of the SD region in different salmonid species by FISH, data supporting lack of homology.

The currently available information on the composition and architecture of model fish genomes advocates for their use to obtain data on the main SD regions of other fish species through comparative genomics (Paterson, 2006; Pennisi, 2007). For this, the availability of markers (usually, microsatellites and SNP) arranged in genetic maps by their co-segregation frequency is essential. These markers can be compared against model fish genomes to look for significant homology, and thus establishing conserved evolutionary tracts at each linkage group defined by two or more adjacent markers (syntenies). Remarkably, high syntenic conservation has been observed within fish, each LG of the investigated species showing a single counterpart in model species (Kasahara et al., 2007; Kai et al., 2011; Bouza et al., submitted). Despite that minor reorganizations have taken place within each LG, data suggests that gene sets on chromosomes have hardly changed along fish evolution. This facilitates the search of genes from model genomes in the regions of interest to identify SD candidate genes (Sarropoulou et al., 2008; Martínez et al., 2009; Loukovitis et al., 2011). Non-model species can also be used as a bridge to connect genomic information between nonmodel species. The so-called stepping stone approach has allowed the establishment of the correspondence between the turbot SD LG5 and the tilapia LG23, where *Dmrta2* and *Amh* were identified as SD candidates, because both LGs were syntenic to Tetraodon LG1, medaka LG4, and stickleback LG8. Thus, *Dmrta2* and *Amh* were strongly considered for mapping in turbot as putative SD candidates (Martínez et al., 2009). Loukovitis et al. (2011) also used this approach to establish the correspondence between European sea bass and Asian sea bass with gilthead seabream LGs to localize suggestive growth QTL in this species that could be related with sex determination.

Genomic Approaches to Study Sex Differentiation

Functional genomics is progressively playing a major role to ascertain the genetic basis of sex determination and differentiation since the arrival of accurate and custom-designed oligo-microarrays specifically designed for different fish species (Ferraresso et al., 2010; Leder et al., 2010; Millán et al., 2010). With this approach, relatively accurate expression patterns can be obtained for sex-associated genes (Leder et al., 2010) or during the formation of the gonad to identify up- or downregulated genes correlated with crucial differentiation events (Baron et al., 2005; Piferrer et al., unpublished data). Because of this, the development of expressed sequence tag (EST)

databases from gonads and brain (including the pituitary), the main reproduction-related organs, using the classical Sanger approach or the more recent NGS technologies, is essential (Pardo et al., 2008). Usually, oligo-probes are designed on a gene-specific region, which avoids cross-hybridization with similar genes of the same family (paralogous genes). The 5′ or, more frequently, the longer 3′-untranslated regions are the most common choice, because of their lower evolutionary conservation than coding regions. Sanger sequencing from cDNA libraries has the advantage of an oriented cloning and sequencing, which facilitates the knowledge of the sense strand for microarray oligonucleotide design. In addition, the physical availability of the whole gene clone permits its complete sequencing, if necessary.

NGS sequences are not oriented and their libraries are not cloned in bacterial hosts. This prevents the identification of the sense strand in nonannotated sequences and physical DNA clones are not available. However, NGS techniques render such amount of information that largely balances any drawback, and this technology has been used to directly identify sex-specific expressed genes such as *dmrt1* y *tra-1* in sturgeon (Hale et al., 2010). Finally, the cDNA AFLP technique has been used to identify relevant genes related to sex determination or sex differentiation. This technique relies on the application of the AFLP protocol on cDNA samples of the gonad tissue at different times of development to identify male or female specific bands, which are subsequently sequenced and annotated. Taboada et al. (2012) was able to identify sex-specific expressed genes in gonads of turbot using this approach.

The approaches taken to study the genomics of fish sex determination and differentiation depend, to a large extent, on whether monosex populations are available. These monosex populations have been achieved in species with a XX/XY system of sex determination, such as the rainbow trout or Nile tilapia, by crossing normal females with sex-reverse genotypic females into phenotypic males with androgen

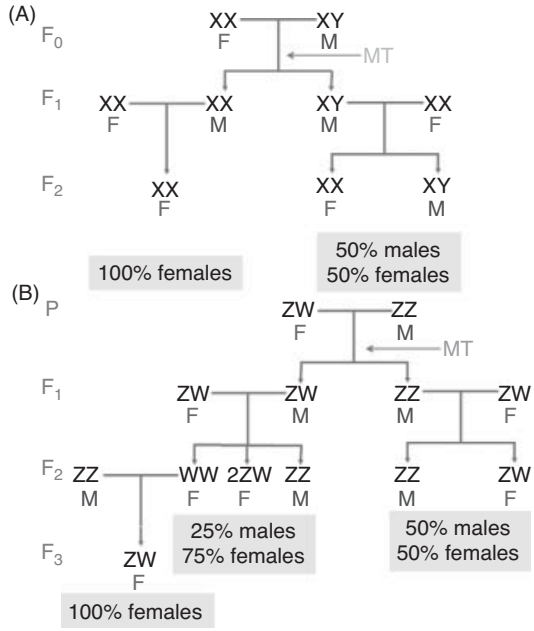

Figure 8.3 Endocrine methods to produce all-female stocks. Diagrams showing the indirect method of feminization by androgen treatment suitable for (A) species with female homogamety and (B) species with male homogamety, assuming the viability of the WW genotype. M, male; F, female; MT, 17α-methyltestosterone. See color insert.

treatment (Figure 8.3A; Piferrer, 2001). The resulting progeny is all female. In species with a ZW/ZZ system of sex determination, a similar approach is also feasible but it takes more time, even if fish with the WW genotype are viable (Figure 8.3B). This provides an excellent tool to study the expression of genes related to a particular sex because during development one can sample a population of known sex well before the sexual differentiation of the gonads.

Recently, with the identification of the SD system (ZW/ZZ) of the turbot, plus its SD region (Martínez et al., 2009), it is expected that with the appropriate endocrine therapy (Piferrer, 2001), the possibility of producing monosex populations will soon be feasible.

Likewise, the strategies available for the transcriptomic study of the gonads during sex differentiation are also related to the type of SD

system. By virtue of the production of monosex populations, as described above, species with a simple XX/XY or ZW/ZZ SD system are the most amenable to study since RNA samples can be obtained in populations of the known genotypic sex not only after but, most important, during and even before the first signs of sex differentiation (Figure 8.4A). In the rainbow trout, for example, this enabled not only the identification of sex-related genes during sex differentiation but also a candidate gene for salmonid sex determination (Yano et al., 2011).

In species where (a) an unknown SD system, (b) known SD system but without monosex populations available, or (c) with SD systems other than monofactorial, there is no other choice than to sample undifferentiated fish of unknown sex genotype and to compare gene expression profiles with those of differentiating males and females and differentiated males and females (Figure 8.4B). This strategy has been applied to study the gonadal transcriptome of males vs. females in the Senegalese sole (J. Viñas and F. Piferrer, unpublished observations) and in the turbot (L. Ribas et al., unpublished observations).

Finally, in species where sex determination is a combination of genetic and environmental influences, such as the European sea bass (polygenic with temperature influences; Vandeputte et al., 2007) or the pejerrey (TSD; Karube et al., 2007), a similar scenario exists to the previous case, but, in order to obtain a reliable information on the interplay between genetics and environment, several sampling schemes under different levels of the main or suspected environmental variable should be considered (Figure 8.4C).

Until recently, the study of sex determination and differentiation has been carried out by examining one or a few genes in what is usually termed the "candidate gene approach," i.e., the study of a gene or a set of genes in a given species, known to play a certain role in other species, including phylogentically distant ones. The objectives were to determine if such a role

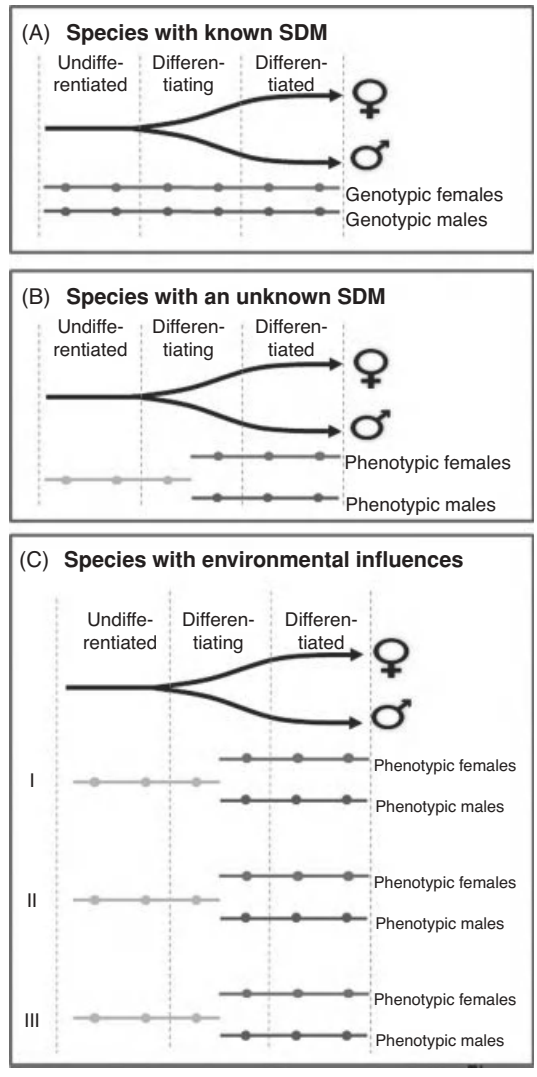

Figure 8.4 Strategies for the transcriptomic analysis of sex differentiation in fish. (A) species with known sex-determining mechanism (SDM); (B) species with an unknown SDM; and (C) species with environmental influences. RNA is harvested from genotypic females (red line), genotypic males (blue line), and undifferentiated fish (green line) at different times (dots) during development. In (C), I, II, and III indicate that the sampling has to be carried including fish reared under different environmental conditions. See color insert.

Table 8.2 Summary of some studies on fish gonadal development using genomic approaches.

Species	Monosex	Stage	Method	Verification	Reference
Guppy	No	Adults	454 Titanium	PCR and RNA-seq	Fraser et al., 2011
Largemouth bass	No	Adults	NGS and microarray	qPCR	Garcia-Reyero et al., 2008
Rainbow trout	Yes	Juvenile	Macroarray, microarray	qPCR	Baron et al., 2007, 2008
Senegalese sole	No	Adult female	Microarray	ISH and qPCR	Tingaud-Sequeira et al., 2009
Sturgeon	No	Juvenile and adults	454 GS		Hale et al., 2009
Sturgeon	No	Adults	454 GS and Titanium	qPCR	Hale et al., 2010
Nile tilapia	Yes	Larvae and fry	qPCR		Ijiri et al., 2008
Nile tilapia	Yes	Adult	Cell transfections, transgenesis	EMSA, ISH	Wang et al., 2010; Wang (2011)
Pejerrey	No	Juveniles	Microarray	qPCR	Fernandino et al., 2011
Xiphophorus	No	Adults	454 Titanium	qPCR	Zhang et al., 2011
Zebrafish	Transgenic	Fry	IHC		Wang et al., 2007b
Zebrafish	No	Adults	Microarrays	ISH and qPCR	Sreenivasan et al., 2008
Zebrafish	No	Adults	Microarrays	qPCR	Small et al., 2009

Notes and abbreviations: 454 Titanium and 454 GS are commercial products from Roche; EMSA, electrophoretic mobility shift assay; NGS, next-generation sequencing; IHC, immunohistochemistry; ISH, in situ hybridization; qPCR, real-time, quantitative PCR.

was conserved or not. Since several years ago, research in this area has also benefited from the development of microarrays and, recently, of NGS. These technology platforms literally allow obtaining millions of sequences of interest. Table 8.2 summarizes some of the studies on fish gonadal development using genomic approaches.

One aspect worth considering is the timing of RNA harvest in relation to the process being studied. Regardless of whether sex determination and differentiation are considered two separate processes following a strict sequence or they are considered globally (e.g., Uller and Helanterä, 2011), they are developmental processes that lead to the formation and differentiation of the gonads over a certain period. Thus, when investigating the genes implicated, one must ask whether observed changes in gene expression reflect the cause or the consequence of a given developmental pathway. This is especially relevant if a treatment (e.g., hormones, temperature) is applied in an attempt to determine the consequences of such treatment on the process. RNA harvest during early sex differentiation or during the treatment being applied will measure immediate effects of the treatment, which may not be relevant in the long term for the question being asked. RNA harvest right at the end of treatment will provide information on the direct consequence of the treatment. The disadvantages with such an approach are the requirement for noncontaminated tissues; in real cases, because

of the developmental dynamics of the gonads, the gonadal tissue will probably be dissected out along with other non-gonadal tissues, which compromises specificity. One way to alleviate this problem is to use germs cells labeled with GFP to aid in the isolation of gonadal tissues. Another problem is that possible sex-related effects cannot be known. In addition, the general lower level of gene expression early in development may hamper finding differences. In contrast, RNA harvest after both the process being studied and the treatment being applied to modify the process are completed has the advantage in that, because the gonads have had time to develop, they are easier to dissect out, such that the effects can be studied separately for each sex and that problems of low gene expression are no longer found. However, this second approach also has a major drawback, i.e., that the measured changes may be the consequence rather than the cause. Another potential problem is the risk of losing the capability of measuring the effects if those happen to be reversible.

Species with Monosex Populations

In species with a monofactorial system of sex determination, such as XX/XY or ZW/ZZ, the production of monosex stocks is feasible. One such species is the rainbow trout, where a great deal of work has been carried out to study genes mainly implicated in sex differentiation. Transcriptomic studies of a hundred of candidate genes responsible for gonad differentiation in all-female populations of rainbow trout revealed that masculinization by androgen (11ß-hydroxyandrostenedione) treatment involved a de-differentiating process in the granulosa cells of differentiating females (Baron et al., 2007, 2008). The hormonal masculinization with androgen first repressed genes related to granulosa cells involved in early ovarian differentiation (e.g., *foxl2*, *cyp19*a) and then decreased other key genes involved in the early oogenesis process (e.g., *gdf9*, *fshb*, *lhb*). In addition, some Sertoli cell markers were upregulated (e.g., *sox9*, *dmrt1*), although some Leydig cell markers were downregulated (e.g., *star*, *cyp11*). Interestingly, when masculinization was achieved by inhibition of *cyp19*a, Sertoli and Leydig cell markers were restored, indicating that masculinization by inhibition of estrogen synthesis resembles more the natural physiological process of masculinization than that achieved by direct androgen treatment (Vizziano et al., 2008). In contrast, when feminization was induced by estrogen treatment, it was found that, as expected, many genes related with early ovarian differentiation, particularly *foxl2*, were upregulated. However, estrogen treatment did not repress genes involved in testis differentiation such as *dmrt1* or *sox9a1*, indicating that what is essential for female differentiation is the *upregulation* of a set of ovarian-specific genes (Vizziano-Cantonnet et al., 2008).

Also, in rainbow trout, Cavileer et al. (2009) used a custom microarray and a strategy based on identifying strong associations in gene expression patterns between known sex differentiation genes (target genes) and novel genes (target-associated genes) previously not allied with sex differentiation in fishes. With this approach, several novel genes were identified in the gonads of embryonic female and male rainbow trout that could be involved in sex-specific differentiation pathways in this fish (Cavileer et al., 2009).

In Nile tilapia, another species where monosex populations can be created, the gonadal expression of *foxl2* and *cyp19a1a* in XX gonads and *dmrt1* in XY gonads at early stages of development (5–6 days after hatching) was found to be critical for undifferentiated gonads to differentiate into ovary or testis, respectively (Ijiri et al., 2008). Other steroidogenic enzymes necessary for the synthesis of estrogens were detected during early stages of gonad differentiation, suggesting that E_2 plays a critical role in ovarian differentiation in tilapia. Recently, functional molecular studies *in vitro* and *in vivo* in this species revealed that *dmrt1* could directly bind to the aromatase promoter. The

transgenic fish overexpressing *dmrt1* had decreased aromatase transcription levels in gonads and consequently lower E_2 plasma levels (Wang et al., 2010). In tilapia, transcriptomic analysis in the gonads of monosex fish revealed a total of 22,385 ESTs in the ovary and testis (Wang et al., 2011). Furthermore, approximately 70,000 sequences from gonadal samples were identified from 3-month-old Nile tilapia, with more genes being expressed at comparatively higher levels in the testis than in the ovary (Huang et al., 2011).

Species without Monosex Populations

The zebrafish (*Danio rerio*) has become an important vertebrate model for basic and biomedical research. This model is also contributing to decipher various aspects of fish reproductive biology, especially when sex differentiation is analyzed at the whole gonad transcriptome level (Li et al., 2004). However, the processes of sex determination and sex differentiation in zebrafish are not well understood. During the first stages of gonad development, primordial germ cells migrate to the genital ridge and their presence is required for the formation of the female gonads. If their presence is inhibited in various ways, the absence of primordial germ cells (PGC) will lead to testis differentiation (Siegfried and Nusslein-Volhard, 2008; Siegfried, 2010). Approximately 24 days postfertilization, juvenile ovaries either enter an apoptotic pathway, which stops ovarian development and testis development follows, or continue with ovarian differentiation depending upon the genetic constitution (Orban et al., 2009). Studies carried out with steroid-treated populations of zebrafish have led to the hypothesis that testis and ovarian differentiation is supported by an underlying ZZ/ZW chromosomal system (Tong et al., 2010). However, steroid-treated populations should be used with caution, since results may not reflect the normal process of sex differentiation. Furthermore, the environmental component regulating sex determination, still poorly defined nowadays in this species, needs to be also taken into consideration (Ospina-Álvarez and Piferrer, 2008; Orban et al., 2009). Analysis of the transcriptome of the zebrafish ovary and testis during gonad differentiation was achieved by using a specific microarray with over 47,000 ESTs (Li et al., 2004). Later, other transcriptomic analysis provided 116,638 gonad-derived zebrafish ESTs and revealed novel genes with sexually dimorphic expression (Sreenivasan et al., 2008), also showing a higher abundance of transcripts in male than in female gonads. Males also had many more expression differences between body and gonads than did females (Small et al., 2009). Recent proteomic studies provided the most comprehensive list of proteins expressed in mature zebrafish gonads, establishing the basis to elucidate processes occurring during fish gonad development from a protein-based perspective (Groh et al., 2011).

In species with environmental influences on sex determination and differentiation, ideally, experiments to study gene expression patterns during sex differentiation should be carried out under different levels of the main environmental variable, e.g., temperature in species with TSD (Figure 8.4C). This, however, is not always feasible and increases experimental costs significantly. In the pejerrey, utilization of a medaka microarray has enabled to identify putative genes involved in sex differentiation in animals reared at male- and female-producing temperatures (Fernandino et al., 2011).

From a pure experimental point of view, there are several potential sources of environmental influences that need to be under control in order to avoid confounding results when studying gene expression levels (Hodgins-Davis and Townsend, 2009; Table 8.3). One of them is effect of the developmental environment, typically in the range of weeks to years. Size is positively correlated with development. However, using size as a proxy for development may give confounding effects if two individuals attain the same size at different ages, consequence of different developmental rates. Another source is

Table 8.3 Potential problems according to the sources of gene expression variation in functional genomics.

Main source	Temporal scale	Potential problems	Consequences
Developmental environment	Weeks–years	Size taken as a proxy for developmental stage	Individuals of the same size may have experienced different developmental rates
Immediate environment	Hours–days	Differences in the level of stimulus or environmental factor	Actual mRNA levels may have integrated effects prior to the start of the experiment
mRNA harvest	Minutes–hours	Variations in: harvest procedure, harvest technique, sample storage, RNA extraction, RT-cDNA synthesis, labeling, hybridization	Introduction of technical noise in measured mRNA levels

the effects of the so-called immediate environment, usually in the range of hours to days. Comparing mRNA levels of a population with a certain level of stimulus against a control population will give different results depending on the immediate environment prior to the start of the experiment. Finally, another source of variation is the effects of RNA harvest, usually in the range of minutes to hours. Many factors affect mRNA levels, some of them need to be taken care by the experimenter, while others usually are dealt with by the platform or service (e.g., microarray platform).

Growth–Sex Differentiation Relationships

There are sources of variation directly linked with the particular biology of the species under consideration. One clear example of genotype × environment interaction concerns the European sea bass, where sex ratios are the product of both genetic (polygenic sex determination mechanism; Vandeputte et al., 2007) and environmental (temperature) influences (Piferrer et al., 2005). In this species, several studies have shown that there is an early association between growth rates and sex (Blázquez et al., 1999), being females among the largest fish and males among the smallest ones (Blázquez et al., 1999; Saillant et al., 2001; Vandeputte et al., 2007). In addition, sexual growth dimorphism is present at the time of marketing when animals reach 300–400 g (Saillant et al., 2001; Navarro-Martin et al., 2009a). Repetitive size-grading of a European sea bass population at early stages generates one female-dominant population with the largest fish and one male-dominant population with the smallest ones (Papadaki et al., 2005). Female-dominant populations were also obtained after a single size-grading (Koumoundouros et al., 2002; Saillant et al., 2003), suggesting an early association between growth rates and sex phenotype.

Studies in mammals have revealed the importance of a threshold in gonad development that needs to be reached before a specific time. Whether this threshold is reached or not influences gonadal fate (Mittwoch, 1989). A similar principle has been suggested for fishes (Kraak and Delooze, 1993). In addition, development rate and the amount of cells in the undifferentiated primordium can influence the actual factor determining the subsequent gonad development (Baroiller et al., 2009a). Thus, sex would at least partially depend on growth. In fish, in general, studies with different species such as the eel (*Anguilla anguilla*; Colombo et al., 1984; Roncarati et al., 1997), the roach (*Rutilus rutilus*; Paull et al.,

2009), and the zebrafish (Lawrence et al., 2008) provided evidence for this relationship between early high growth and female differentiation. However, studies with steroid-treated European sea bass showed no differences in growth neither between normal females and E_2-feminized females (Saillant et al., 2001), nor between normal males and Fadrozole (an aromatase inhibitor)-masculinized males (Navarro-Martin et al., 2009b), suggesting that growth depends on phenotypic sex.

Recent experiments with the European sea bass have shown that reduced growth with or without previous size-grading of the population during the sex differentiation period (8–12 cm standard length) did not affect sexual differentiation. Rather, the final sex ratios were related to growth rates at the time of the size-grading, indicating that the association between sex and growth rates is established earlier than previously thought (N. Díaz et al., unpublished observations). Furthermore, this was independent of the occurrence of compensatory growth, a phenomena where growth rates increase and size converge between fish previously subjected to different growth rates (Ali et al., 2003; Jobling, 2010). To explore further this relationship, a sea bass microarray has been used to compare gene expression in the differentiating testis of fast vs. slow growing males. Preliminary results (Figure 8.5) indicate differences in expression of important genes related to reproduction such as the anti-Müllerian hormone, *dmrt1* or *cyp19a*. Together, these results illustrate not only that the final sex ratio of the population is established well before the first signs in sex differentiation are visible, but also that it is not affected by growth rates. It should be noted that in experiments on species where environmental factors influence sex ratios, caution in the experimental design should be taken. Thus, when looking for genotype × environment effects is usually advisable to reduce the number of genetic (families, strains) and environmental conditions but to maintain the highest possible number of individuals rather than to increase the number of combinations at the expense of reducing sample sizes.

Contribution of Epigenetics

Epigenetics is a very active area of research nowadays. A major epigenetic mechanism involves changes in the methylation patterns of DNA. When these changes occur in CpG nucleotides located within the promoter region of genes, they significantly contribute to changes in gene expression level. Epigenetic mechanisms involving DNA methylation have been implicated in the normal development of the zebrafish (Mhanni and McGowan, 2004), the activation and silencing of the SD gene, *SrY*, in mammals (Nishino et al., 2004), and in the responses to temperature in plants (Sung and Amasino, 2004). Epigenetic mechanisms have also been implicated in the reproductive alterations following exposure to pollutants (Zama and Uzumcu, 2010).

It has been argued that embryonic and larval fishes are particularly well suited for epigenetic research in a variety of aspects including, but not limited to, feeding behavior, food conversion, and hypoxic tolerance (Burggren and Blank, 2009). Technical advantages of fish include external fertilization, relatively high fecundity, and short life cycle. An example of an epigenetic effect is the observation that adult zebrafish chronically exposed to 2–4 weeks of moderate hypoxia and then returned to normoxia subsequently produced offspring that exhibited enhanced hypoxic resistance. Thus, it is not difficult to ascertain that these aspects are of extraordinary importance in the context of modern, intensive fish farming where conditions may be suboptimal because of high rearing densities. The zebrafish and the sea bass are two of the richest species in terms of genomic resources (Kuhl et al., 2010), and thus two suitable species where to begin to integrate epigenetics research for aquaculture applications.

Gorelick (2003) proposed that in animals with TSD, sex differences were first determined

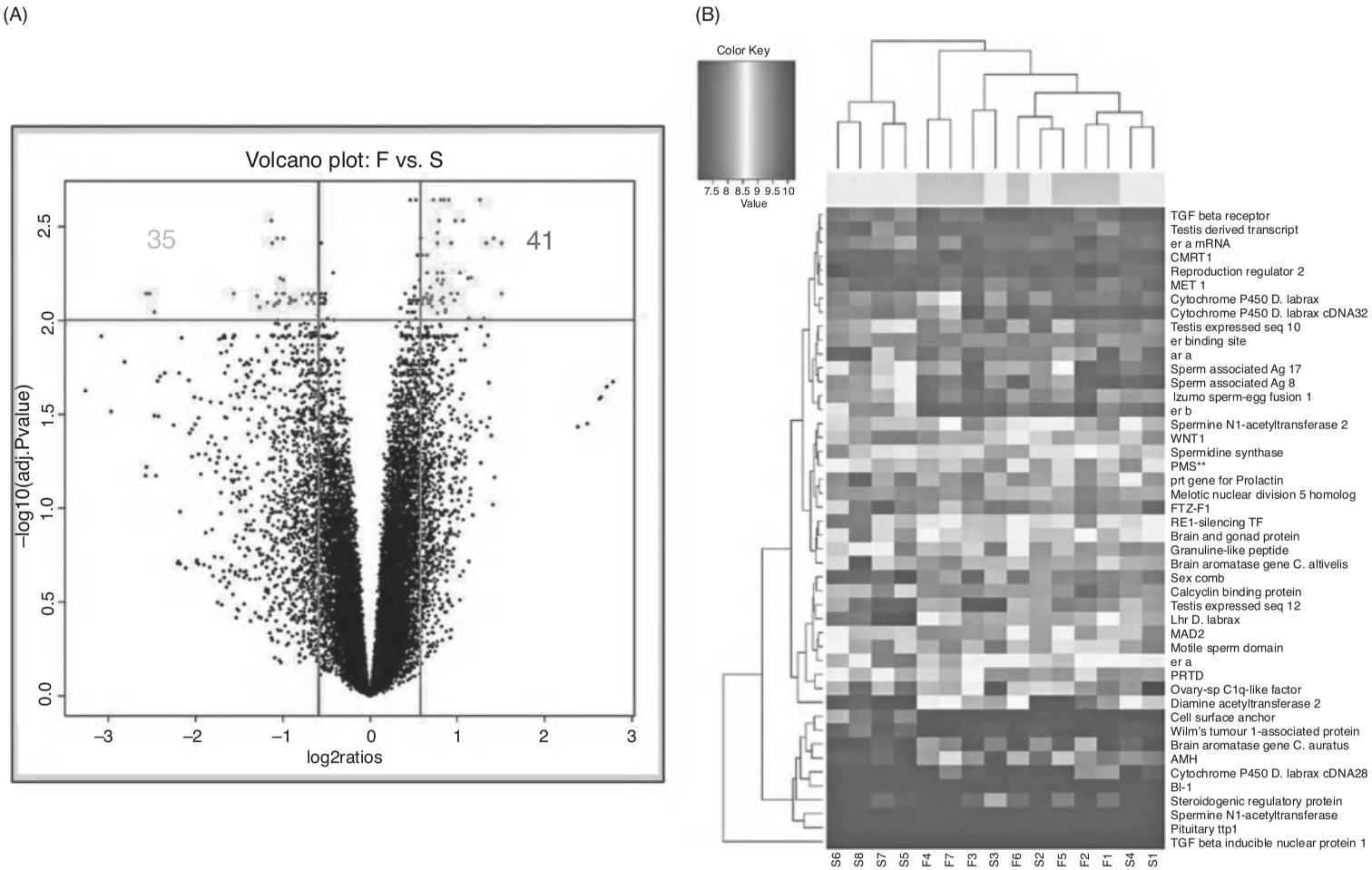

Figure 8.5 Transcriptomic analysis of sexually undifferentiated European sea bass sampled at 133 days postfertilization. (A) Volcano plot of genes being downregulated (blue) or upregulated (red) at a fold change > 1.5 with $p < 0.01$ between large (L) and small (S) European sea bass. (B) Heat map of reproduction-related genes between L and small S fish (Díaz et al., unpublished data). See color insert.

by different patterns in DNA methylation and also that environmental variations may change those patterns. Earlier studies in our laboratory showed that *cyp19a* not only was an early marker of ovarian differentiation but also that the expression of this gene was inhibited by high temperature (Blázquez et al., 2009). However, the mechanism connecting external temperature and aromatase, although essential for sex differentiation in species with TSD including reptiles and fish, has remained elusive (Lance, 2009; Luckenbach et al., 2009).

In the European sea bass, the masculinizing effects of high temperature are maximal during early development (Navarro-Martin et al., 2009a), not only well before the sexual differentiation of the gonads but even before the formation of the gonadal ridges themselves. Thus, it was conceivable that an epigenetic mechanism could be part of the sex ratio response to temperature in species where this environmental cue is able to modulate sex ratios, a question never before explored. Research on this subject with one-year-old sea bass showed that the *cyp19a* promoter was hypermethylated in males when compared with females, in agreement with the well-established lower levels of *cyp19a* expression observed in testes than in ovaries. Furthermore, we also found that high temperature increased the methylation levels of *cyp19a* in both sexes. Finally, luciferase assays confirmed that methylation of the promoter causes the *in vitro* repression of transcription, suggesting that *cyp19a* promoter methylation is the mechanism by which *cyp19a* transcription is silenced in developing males during sex differentiation, preventing the differentiation of the undifferentiated gonad into an ovary (Navarro-Martín et al., 2011).

The analysis of epigenetic changes in response to varying environment will be very relevant in the coming years. The analysis at the whole genome scale still poses important challenges (Laird, 2010). However, we expect that only at this level, the most significant contributions brought by integrating epigenetics will be made.

Concluding Remarks and Future Prospects

Fish sex ratios are the result of a complex interaction between genetic, biochemical, and environmental interactions. The ultimate result of these interactions at the individual level is gender: male or female. However, at the population level, the combination of sex determination and differentiation sets the sex ratio. In turn, sex ratios define the reproductive capacity of populations and, if sex growth dimorphism exists, also the growth characteristics, something very important in an aquaculture context. Furthermore, despite some efforts have been made in this direction, the relationship between fitness, sex, and growth deserves further research. To this end, NGS techniques can help to find SD genes and molecular markers of sex differentiation in fish, by far not only the most diverse group of vertebrates in terms of number of species but also, specially, in terms of reproductive strategies and SD mechanisms.

In many species of fish, the existence of strong G $\times$ E interactions may lead to complex reaction norms connecting variation of a given environmental factor and phenotype (sex ratios). In this context, it is envisaged that epigenetic approaches will become more common and will help to understand how the environment controls gene expression related to production traits.

Finally, the development of molecular probes to identify sex or the establishment of molecular signatures characteristic of a given phenotype can have an immediate application to the fish-farming industry for broodstock selection and monosex production for better performance.

Acknowledgments

ND was supported by a predoctoral scholarship from the Spanish Ministry of Science (MICINN) program FPI (ref. BES-2007-14273); LR was supported by a postdoctoral

contract. Research was supported by MICINN grants SexGene (AGL2006-01359) and Epigen-Aqua (AGL2010-15939) to FP; Aquagenomics (CDS2007-002) to both FP and PM, and Xunta de Galicia (10MMA200027PR) to AV). The authors want to clearly state that part of the contents of this chapter have been adopted from a review submitted by Piferrer et al. (2012).

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Chapter 9

Functional Genomics of Stress: Molecular Biomarkers for Evaluating Fish CNS Activity

Chiara Tognoli, Rosalba Gornati, Marco Saroglia, Genciana Terova, and Giovanni Bernardini

Abstract: Aquaculture currently contributes almost half of the fish consumed by the human population and is growing more rapidly than other animal food production sectors. Use of molecular techniques for the monitoring of stress levels using early indicators, such as molecular biomarkers, can bring considerable benefits to the quantity and quality of aquaculture production and improve the welfare of reared animals. Moreover, in recent years, some teleosts, such as zebrafish and pufferfish, have become model organisms, and the knowledge gathered about them can be applied to further improve rearing and husbandry of species of economic interest, which could, in turn, become animal models themselves. Fish, whose brain organization is similar to that of higher vertebrates, are generally considered free of emotional reactions and consequently can become an interesting model for addressing general questions on stress. Stress alters brain function, which may precipitate mood disorders. The neurotrophin brain-derived neurotrophic factor (BDNF) was recently shown to be involved in stress-induced adaptation. BDNF is a key regulator of neuronal plasticity and adaptive processes. Regulation of BDNF is complex and may reflect not only stress-specific mechanisms, but also hormonal and emotional responses. In this chapter, we discuss stress responses of sea bass, *Dicentrarchus labrax*, a marine teleost species of great interest for aquaculture. Acute stress causes downregulation of BDNF mRNA splicing variants 1*c* and 1*d*. Acute stress also induces a significant increase in proBDNF levels and reduction in mature BDNF, suggesting that the regulation of proBDNF proteolytic processing has been altered. Here, proteolytic regulation of BDNF is simplified since the pro28kDa form, generated by the SKI-1 protease in mammals, is absent in fish. The highly predictable proBDNF/totBDNF ratio for stress in lower vertebrates indicates that BDNF processing represents a central mechanism in adapting to stress and predicts that a similar regulation of pro/mature BDNF has likely been conserved throughout evolution of vertebrates from fish to humans.

Introduction

Aquaculture currently contributes almost half of the fish consumed by the human population. This sector has been growing extensively in the last 50 years and continues to expand more rapidly than any other animal food-producing

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

sector (FAO, 2009). The recent introduction of genome-based technologies in addition to the more traditional biotechnological methods has improved the potential to significantly increase aquaculture production in the world. The new approaches include genome projects, cDNA microarray expression analysis, functional genomics, novel transgenic technologies, proteome analysis, and metabolomics. All these approaches might be applied to gain a better understanding of the biology of aquaculture species, which, in turn, can improve growth and cost effectiveness, resistance to environment and pathogens, broodstock quality, and reproduction control, and help create new and better products (Melamed et al., 2002).

Growth enhancement is an important goal in aquaculture, especially when it can be achieved through more economic and faster manipulations than selective breeding methods. One of the ways to increase growth rates in animals, originally demonstrated in mice (Palmiter et al., 1982), is to introduce additional copies of growth hormone gene into the animal genome. For instance, transgenic salmonids possessing an "all-fish" gene construct consisting of antifreeze protein (AFP) promoter and growth hormone cDNA showed a dramatic growth enhancement (Du et al., 1992; Devlin et al., 1994). They appear healthy and some produce second- and third-generation transgenic offspring that maintain the enhanced growth phenotype (Saunders et al., 1998).

An interesting phenomenon studied in intensive aquaculture as a means of enhancing growth rates is "compensatory growth," an exceptionally fast growth that occurs in fish after periods of fasting (see Chapter 5 for details). The mechanisms by which food intake activates an increase in somatic growth, especially in muscle, are complex and not yet fully understood. Terova et al. (2006, 2007a) identified three genes involved in compensatory growth in sea bass (*Dicentrarchus labrax*): insulin-like growth factor I and II, which are potent mitogens, and myostatin, a factor important for skeletal muscle growth.

The molecular approaches currently employed to increase resistance to viral and bacterial pathogens are mostly based on DNA vaccines and antimicrobial agents. With DNA vaccines, naked DNA encoding part of the antigen that induces the production of antibodies is injected into fish. DNA vaccines have been studied in a number of fish (Lorenzen et al., 1999; Traxler et al., 1999). Antimicrobial proteins, like lysozymes, can be used to target the nonspecific immune response (Andreu and Rivas, 1999). Alternatively, transgenic fish carrying genes encoding antimicrobial peptides can be created.

Molecular techniques can also be used to increase resistance to adverse environmental conditions, such as excessively cold temperatures in fish. Some marine teleosts possess high levels of serum AFP or antifreeze glycoprotein, which effectively reduce the freezing temperature by preventing the growth of ice crystals. The gene encoding the liver AFP from winter flounder was successfully introduced into the genome of Atlantic salmon (Hew et al., 1999). Indeed, developing stocks that harbor this gene would clearly offer a major benefit for commercial aquaculture in countries where winter temperatures often border the physiological limits of these species.

Aquaculture and Model Organisms

In recent years, teleost species have increasingly been used as vertebrate model organisms to study the genetics underlying development, normal body functions, and diseases, with a parallel increase in new tools and methodologies. Transferring knowledge from models to the species of interest for aquaculture is an important channel to further improve rearing and husbandry traits, such as growth rate, stress resistance, and disease resistance (Dahm and Geisler, 2006).

The most popular fish model is the freshwater teleost zebrafish, *Danio rerio*, which has become an experimental model for

embryogenesis, organogenesis, vertebrate development (Love et al., 2004), and human diseases such as cancer or neurodegenerative disorders (Amatruda and Patton, 2008). Medaka (*Oryzias latipes*) represents another model organism employed to study aspects of embryonic development (Wittbrodt et al., 2002). Behavioral or evolutionary developmental biology studies have been performed on stickleback, *Gasterosteus aculeatus* (Tickle and Cole, 2004); the two pufferfish, *Takifugu rubripes* and *Tetraodon nigroviridis*, have become important organisms for understanding genome architecture, organization, and function because of the relatively small size and simple organization of their genomes.

Other aquatic animal models have been used in biomedical research, and many of them are directly relevant for understanding the physiology, genetics, anatomy, and pathology of human disease processes (Schmale, 2004). In this regard, the green swordtail (*Xiphophorus* sp.) can be cited as a well-established model for human melanoma (Meierjohann et al., 2004) and the adult brain of the Atlantic salmon (*Salmo salar*) as an experimental model for neuronal tissue regeneration after injury (Zupanc et al., 2006). Some teleost species are also suitable for environmental monitoring: besides zebrafish, the rare minnow (*Gobiocypris rarus*) has recently been selected as a model for aquatic toxicological studies (Zhang et al., 2008); exposures to heavy metals such as zinc and cadmium, biological toxins, or more undefined pollutant mixtures have been monitored through proteomic studies in different fish species (Forné et al., 2010).

Genomic Resources for Fish

A major drawback for applying molecular techniques in aquaculture is the lack of genetic information about the species of commercial interest as compared with the model organisms, even though these resources are being expanded. A recent review summarized current information about fish nucleotides (Oleksiak, 2010). Presently, five fish genome projects are in progress, which comprise the aforementioned zebrafish (*D. rerio*), medaka (*O. latipes*), *T. rubripes*, *T. nigroviridis,* and *G. aculeatus*. However, genome projects on Atlantic salmon (*S. salar*) and rainbow trout (*Oncorhyncus mykiss*), which are species of enormous interest in aquaculture, are also underway.

Although nuclear genome projects are limited to some fish species, a wealth of sequence data exists for many more fish species, largely based on expressed sequence tag (EST) projects. ESTs represent a partial sequence of RNA molecules expressed in a cell. Being encoding genes that are actively transcribed, without intron sequences, they can be more informative about the ultimate function of the gene.

ESTs resources represent one of the efforts being undertaken to reduce the gap in our knowledge of farmed species and model organisms. With this aim, our laboratory has, in recent years, established eight cDNA libraries obtained from different tissues of three teleosts: *D. labrax, Perca fluviatilis*, and *Thunnus thynnus* (Chini et al., 2006, 2008; Rossi et al., 2007). Interested readers are referred to Chapter 2 of this book, which provides a comprehensive review of genome resources currently available for aquaculture species.

Molecular Biomarkers for Animal Welfare

Protecting the welfare of the animals is a central requirement of any animal-rearing system, including fish. Animal welfare involves the subjective feelings of animals and the experience of pleasure, pain, frustration, hunger, or other states, and moreover, it is difficult to define and to measure (Dawkins, 1990). Recently, Korte et al. (2007) proposed a new animal welfare concept based on allostasis, which means stability through change, and it has the potential to replace homeostasis as the core model of physiological regulation. Not

constancy or freedoms, but rather capacity to change is crucial for good physical and mental health and good animal welfare.

Stress responses in fish have been broadly characterized as primary, secondary, and tertiary responses. The primary response starts with an increase in neuroendocrine/endocrine factors such as cortisol in the blood. The secondary response comprises various hormone-induced biochemical and physiological effects, which alter hematological parameters such as glucose concentration. The tertiary response is associated with "fish social life," which may result in appetite loss, compromised anabolic processes, reduced reproductive capability, and frequent occurrence of infectious diseases (Terova et al., 2009b). Therefore, stress conditions have traditionally been evaluated by monitoring blood levels of cortisol, hemoglobin, and glucose (Roche and Bogè, 1996). However, these descriptors may not be sufficiently reliable when chronic stress is applied and animal welfare is concerned. It is therefore necessary to search for further parameters that can describe biological stress and animal emotional responses, taking into account the "allostatic concept" (Korte et al., 2007). Molecular biomarkers directly indicate gene activity, and for this reason, they have the characteristics of early indicators useful as biomarkers (Prunet et al., 2012).

The search for molecular markers can be approached by looking for genes whose expression could reasonably be modified by the different farming conditions or genes whose expression can be modified by stress changes, for example, in rearing densities. With differential display, Gornati et al. (2004a) obtained six bands differentially expressed, comparing gene expression of sea bass farmed at different population densities. One of these bands was found to encode for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), a key enzyme for cholesterol synthesis. Quantitative evaluation of HMGCR expression by real-time PCR confirmed an upregulation of transcription at higher population densities (Gornati et al., 2005). In this context, other classical, stress-related genes, such as metallothioneins, heat-shock proteins (Gornati et al., 2004b, 2005), enolase, Na^+/H^+ exchanger 1, c-Fos, glucocorticoid receptor, and glucose transporter 2 (Terova et al., 2005, 2009a; Rimoldi et al., 2009), and genes related to specific stress conditions, such as oxygen fluctuations (HIF-1α) and food deprivation (Terova et al., 2007b, 2008a, 2008b), have been studied.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is the most abundant and widely expressed of the neurotrophins, a family of structurally related proteins required for the development and function of the vertebrate nervous system (Casaccia-Bonnefil et al., 1999; Huang and Reichardt, 2001; Poo, 2001). In the vertebrate brain, BDNF also governs long-lasting changes in synaptic efficacy and morphology (McAllister et al., 1999; Thoenen, 2000; Bramham and Messaoudi, 2005; Bramham, 2007; Lu et al., 2008). Recent studies have suggested that BDNF may be involved in stress-induced adaptation in adults (Marini et al., 2008). Indeed, several types of injury and cell stress affect the expression of BDNF in the mammalian brain; in particular, chronic stress decreases the synthesis of hippocampal BDNF (Smith et al., 1995a, 1995b, 1995c; Nibuya et al., 1999), while acute stress induces complex alterations in the expression of BDNF, including a decrease in the hippocampus and an increase in the prefrontal cortex (Marmigère et al., 2003; Nair et al., 2007; Lee et al., 2008; Fuchikami et al., 2009).

"Stress" is a biological term that refers to the consequences of failure of a human or animal body to adequately respond to environmental stimuli. Stress induction is also used to study alterations in brain functioning leading to mood disorders, which are often precipitated or exacerbated by acute or chronic stressful life events (Gold and Chrousos, 2002; Brown et al., 2003;

Duman and Monteggia, 2006). Stress also involves subjective feelings that are particularly complicated in mammals and primates in which emotional components may have a dominant effect. Alterations in BDNF expression were also found in response to emotions such as anxiety or fear in rodents (Rasmusson et al., 2002), and it has been shown that BDNF affects emotional preferences in humans (Gasic et al., 2009). It remains to be determined how the stress itself or the associated behavioral responses contribute to mediating these changes. From this perspective, fish, whose brain organization is very similar to that of higher vertebrates, but is generally considered free of emotional reactions, is interesting as an animal model of stress.

Neurotrophin Family Members and Their Receptors

In addition to BDNF, the family of neurotrophins includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) and also neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7), which are found only in fish.

All known neurotrophin genes share a common organization: they encode for a pre-pro-protein that is translocated to the endoplasmic reticulum and proteolytically processed to yield the mature protein. Mature neurotrophins form noncovalently associated homodimers that share a highly homologous structure with features such as tertiary folds and cystine knots and that are present in several other growth factors. The specificity of neurotrophin activity is dependent upon the activation of three different tyrosine kinase (Trk) receptors and their downstream signaling cascades: NGF specifically activates TrkA, BDNF, and NT-4/5, which interact with TrkB, whereas NT-3 preferentially binds to Trk-C. NT-6 and NT-7 do not have orthologues in mammals or birds and appear to interact with the same receptors as the mammalian proteins.

The first receptor to be discovered, p75 neurotrophin receptor (p75NTR), was identified as a low-affinity receptor for NGF, but was subsequently shown to bind each of the neurotrophins with a similar affinity. p75NTR is a member of the tumor necrosis receptor superfamily, with an extracellular domain that binds pro-neurotrophins with high affinity to produce a number of cellular responses, including apoptosis.

In mammals, the three members of the Trk subfamily of receptor Trks constitute the second major class of neurotrophin receptors. The neurotrophins dimerize the Trk receptors by binding them, resulting in activation through transphosphorylation of the kinases present in their cytoplasmic domains. Each Trk receptor controls three major signaling pathways that promote neuronal differentiation, including neurite outgrowth, survival, and growth of neurons and other cells, synaptic plasticity, and gene transcription (Lu et al., 2005; Reichardt, 2006; Lanave et al., 2007).

BDNF: Gene Structure, mRNA, and Protein Quantification after Stress

All known vertebrate BDNF genes share a similar, multiple exon organization and encode for a pre-pro-protein that is translocated to the endoplasmic reticulum and proteolytically processed to yield the mature protein (Aid et al., 2007; Pruunsild et al., 2007; Tettamanti et al., 2010). The BDNF genes of the teleosts zebrafish, pufferfish, and sea bass have a similar exon/intron organization and are highly homologous. The *D. labrax* gene spans about 15 Kb (GeneBank accession number FJ711591) and consists of at least five alternative 5′-exons (i.e., 1β, 1*a*, 1*b*, 1*c*, and 1*d*) and one 3′-coding exon (Tognoli et al., 2010). Comparing the structure of BDNF gene in vertebrates, it should be noted that the nomenclature of the different exons of BDNF used for teleosts is different (Heinrich and Pagtakhan, 2004) from that used for

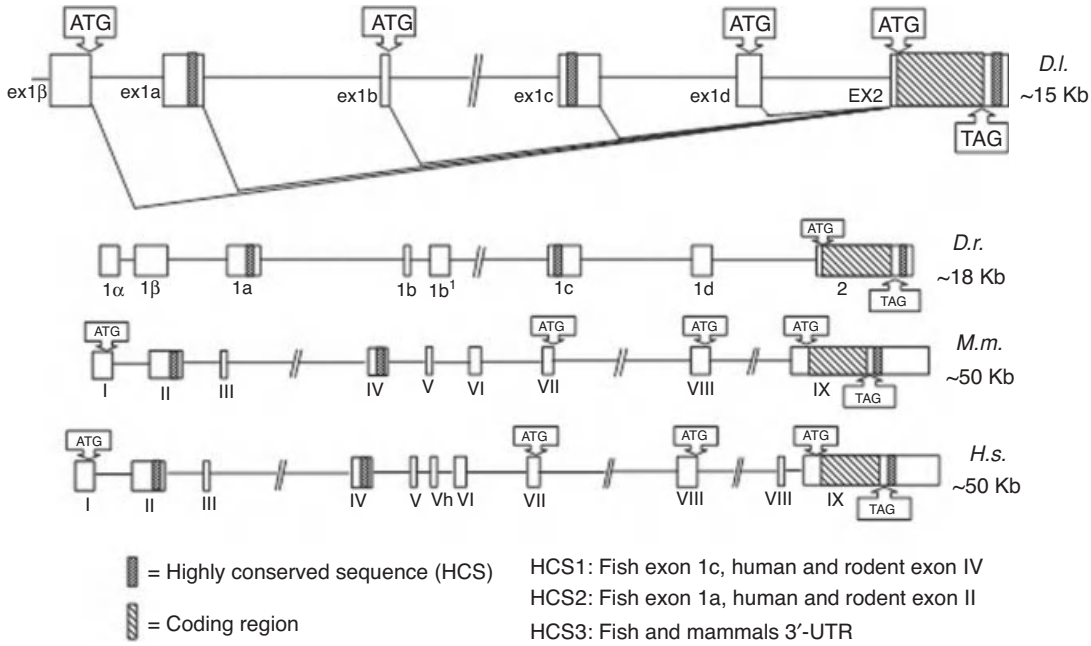

Figure 9.1 Gene organization and of BDNF. Organization and approximate length of BDNF gene in *Dicentrarchus labrax*, *D.l.*; *Danio rerio*, *D.r.*; *Mus musculus*, *M.m.*; *Homo sapiens*, *H.s.*; exons are shown as boxes; and introns as lines. Alternative start codons (ATG) as well as stop codon (TAG) were reported. (Modified from Tognoli et al., 2010.)

mammals, birds, and amphibians (Kidane et al., 2009), and thus the coding sequence of the *D. labrax* gene is in exon 2. The coding sequences are highly conserved as compared with those from other vertebrate species (*D. rerio* 84%, *Fugu rubripes* 91%, *Homo sapiens* 77%, *Mus musculus* 78%, and *Rattus norvegicus* 78%).

Analysis of *D. labrax* BDNF transcripts, carried out both in the early developmental stages and in adult tissues, demonstrated that all alternative upstream exons (1β, 1*a*–1*d*) are spliced to the protein-coding exon 2. This indicates that the structure of *D. labrax* BDNF transcripts is similar to that of other vertebrates. In fact, multiple transcriptional initiation sites and splicing into two-part transcripts can also be found in humans, chimpanzees, dogs, pigs, cats, cows, chickens, frogs, lampreys, zebrafish, and pufferfish (Heinrich and Pagtakhan, 2004). With the exception of exon 1*a* (43%), other exons of the *D. labrax* gene exhibit strong sequence homology with zebrafish and pufferfish genes. Furthermore, exon 1β contains a 170-nt segment that is highly similar to the human exon 1 (75% identity). Three highly conserved segments were found in sea bass BDNF: HCS2 in exon 1*a* that is also present in exon II*c* of mammalian BDNF, HCS1 in exon 1*c* that is also found in mammalian exon IV, and HCS3 in 3′UTR of exon 2 that is also present in the 3′UTR of mammals (Figure 9.1). These results suggest that mammalian exons I, II, and IV (coding for the 5′ untranslated region) evolved early in vertebrate radiation and may play a major role in BDNF activity, while more recently evolved splice variants, including other 5′ exons, may participate in more specialized functions of the BDNF genes, e.g., synaptic plasticity. Further research in this direction could be undertaken to test this hypothesis.

```
    atgaccatcctgttccttactatggttatttcatacttcagttgcatgagagctgcgccc
 1    M  T  I  L  F  L  T  M  V  I  S  Y  F  S  C  M  R  A  A  P
    ctgagagacgccccgggcatgcggggccatcggacggaaggctacttgggcgccgctgcg
21   L  R  D  A  P  G  M  R  G  H  R  T  E  G  Y  L  G  A  A  A
    acggccgcgcgaggccatgggactccacagagtggtggtgggccaggccagcgcggggaa
41   T  A  A  R  G  H  G  T  P  Q  S  G  G  G  P  G  Q  R  G  E
    ctgccctcactcacagacacgtttgagcaggtgatagaggagctgctggaggtggaggga
61   L  P  S  L  T  D  T  F  E  Q  V  I  E  E  L  L  E  V  E  G
    gaggcggcacagctgggacaggggggctgataagagccagggaggtgggggcccatcctct
81   E  A  A  Q  L  G  Q  G  A  D  K  S  Q  G  G  G  G  P  S  S
    gtggtcaccacagaggccaaggatgtcgacctgtacgactcgcgggtgatgatcagcaac
101  V  V  T  T  E  A  K  D  V  D  L  Y  D  S  R  V  M  I  S  N
    caagtgccttttggagccgccgttgctctttcttctggaggaatacaaaaactatctggat
121  Q  V  P  L  E  P  P  L  L  F  L  L  E  E  Y  K  N  Y  L  D
    gccgctaatatgtgcatgagggtgcggcgacactccgatccctcacggcgtggagagctc
141  A  A  N  M  C  M  R  V  R  R  H  S  D  P  S  R  R  G  E  L
    agcgtgtgtgacagtattagccagtgggtgacagctgtggataaaaagacggcaatagac
161  S  V  C  D  S  I  S  Q  W  V  T  A  V  D  K  K  T  A  I  D
    atgtctgggcagacagttaccgtcatggaaaaggtccctgtccccaatggccaactgaag
181  M  S  G  Q  T  V  T  V  M  E  K  V  P  V  P  N  G  Q  L  K
    caatacttttatgagaccaaatgcaaccccatggggtacacaaaggagggtgcagagga
201  Q  Y  F  Y  E  T  K  C  N  P  M  G  Y  T  K  E  G  C  R  G
    atagacaagcggcattataattcccaatgcaggacaacccagtcctacgtgcgagcgctt
221  I  D  K  R  H  Y  N  S  Q  C  R  T  T  Q  S  Y  V  R  A  L
    accatggatagcaaaaagaagattggctggcggtttataggatagacacttcatgtgta
241   T  M  D  S  K  K  K  I  G  W  R  F  I  R  I  D  T  S  C  V
    tgcacattgaccattaagaggggcagatagtgtataaaatgtatagattttattgaagag
261    C  T  L  T  I  K  R  G  R  -
```

Figure 9.2 Protein and cDNA sequence of BDNF. *Dicentrarchus labrax* cDNA sequence and deduced amino acid sequence (GeneBank accession number FJ711591). Amino acid sequence of signal peptide is boxed in white; amino acid sequence for *N*-glycosylation is boxed in black; the cleavage sequence is underlined; and start codon and the mature BDNF are shown in bold. (Modified from Tognoli et al., 2010.)

The protein organization (Figure 9.2) in sea bass is similar to that of other teleosts (Hashimoto and Heinrich, 1997), birds (Maisonpierre et al., 1992), and mammals (Hofer et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1991). Sea bass proBDNF has 87% identity with that of zebrafish and 74–75% with that of mammals. Two regions were >95% identical: the first 20 amino acids at the *N*-terminus, comprising the signal peptide, and 35 amino acids just upstream of the cleavage site, which encodes for the glycosylation consensus site. Interestingly, exons 1β, 1*b*, and 1*d* have an in-frame ATG codon that could be used as translation start sites, leading to the pre-pro-BDNF proteins with longer *N*-termini. However, analysis of the extended *N*-terminal sequences with the prediction program SignalP 3.0 (Bendtsen et al., 2004) indicated that the *N*-termini produced by exons 1β and 1*b* score poorly as signal peptides because of the presence of a putative signal anchor, while the very long sequence produced by exon 1*d* does not encode for a signal peptide.

The post-hatching developmental expression analysis indicated that, although in different amounts, all *D. labrax* BDNF transcripts, except 1*d*/2, are well represented

at all developmental stages, with the 1*c*/2 splice variant showing the highest expression. The distribution of the different BDNF transcripts in adult sea bass is tissue-specific, with all transcripts being mostly expressed in the brain. The splice form 1*c*/2 is also expressed, although at low levels, in all the examined extranervous tissues (liver, kidney, and muscle), while exon 1*b*/2 transcript is found only in the kidney (Table 9.1). Organ-specific expression also holds for most BDNF exons in zebrafish and mammals, suggesting conserved transcriptional regulation among vertebrates (Heinrich and Pagtakhan, 2004; Liu et al., 2006; Aid et al., 2007; Pruunsild et al., 2007; Tettamanti et al., 2010). According to this view, the bioinformatic analysis of *D. labrax* BDNF gene suggests that the region upstream of exon 1*c* potentially contains two responsive elements belonging to the CRE family (cAMP/calcium responsive element-binding protein). These elements also function as responsive elements in BDNF exon IV of rat cortical neurons (Heinrich and Pagtakhan, 2004; Liu et al., 2006) and may be responsible for the higher expression of the isoform 1*c*/2 in adult sea bass tissue. The presence of the HCS1 in fish exon 1*c* and mammalian exon IV supports the likelihood that these two exons are true orthologues. It is noteworthy that zebrafish HCS1 behaves, depending on the sequence context, as an enhancer or a dehancer (Heinrich and Pagtakhan, 2004).

Acute stress induces variations in BDNF transcript expression in the brain of rodents (Marmigère et al., 2003; Nair et al., 2007; Fuchikami et al., 2009). The transcription of exons 1*c* and 1*d* decreases significantly in *D. labrax* as well when fishes are subjected to a brief stressful event. This is consistent with data in rodents, in which a single immobilization stress induces downregulation of exon IV, homologous to fish exons 1*c* (they both contain HCS1), because of decreased histone acetylation at this promoter immediately after acute stress (Fuchikami et al., 2009). A recent study showed that exons of the second promoter clusters (mammalian exons IV–VII, fish 1*c*–*d*) are particularly important for cell survival in response to cytotoxic stress in human neuroblastoma cells (Baj and Tongiorgi, 2009). Thus, activation of promoters upstream of these exons might be related to a rapid adaptive response to various types of stress.

Table 9.1 Expression of BDNF splice variants in different tissues of adult *Dicentrarchus labrax.*

Splice variants	Adult tissues expression pattern			
	Brain	Liver[a]	Kidney[a]	Muscle[a]
1β/2	+	n.d.	n.d.	n.d.
1*a*/2	+[a]	n.d.	n.d.	n.d.
1*b*/2	+/−	n.d.	+/−	n.d.
1*c*/2	+	+/−	+	+
1*d*/2	+[a]	n.d.	n.d.	n.d.

n.d., not detectable.
[a] An aliquot of first PCR product was amplified in a second round of PCR.
Source: Modified from Tognoli et al., 2010.

The analysis of protein levels after acute stress showed that in brain, but not in liver, proBDNF content is significantly increased in the stressed animals. Acute stress profoundly alters the relative amount of proBDNF and mature BDNF, suggesting lower proteolytic activity to generate mature BDNF and thus, an accumulation of the uncleaved product in the sea bass brain. The scatter-plot distribution analysis of the two populations showed that the percentage of proBDNF in the brain of every animal of the stressed group is at least one standard deviation above the mean value of the control group (Figure 9.3). Although the mechanisms by which stress can prevent efficient conversion of proBDNF into mature BDNF are presently unknown, several recent studies have indicated that pro32KDa BDNF has a biological function distinct from that of mature BDNF. Both proBDNF precursor and mature BDNF can be released from neurons (Lee et al., 2001; Yang et al., 2009). While proBDNF binds only to p75 receptor, mature BDNF displays a high affinity to TrkB and a lower affinity to p75 (Chao, 2003). Binding of proBDNF to p75

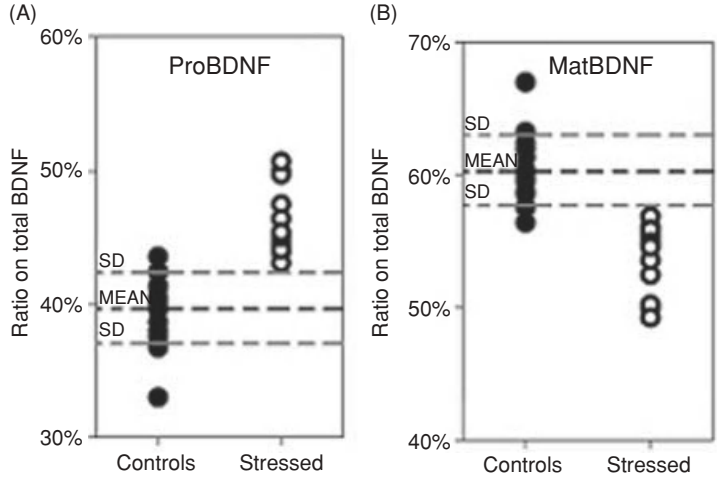

Figure 9.3 Stress significantly increases the percentage of proBDNF while decreasing matBDNF in the brain of stressed sea bass. (A) The scatter plot shows that in the brain of all stressed animals proBDNF are above the mean of controls + 1 SD. (B) Similarly, in all stressed animals, mature BDNF values are below the mean of controls −1 SD. Only two control animals have a proBDNF percentage above the controls' mean + 1 SD for proBDNF or below the mean −1 SD. The *F* values, obtained by one way ANOVA analysis, for proBDNF and mature BDNF were 19.028 and 6.225, respectively. (Modified from Tognoli et al., 2010.)

promotes cell death and attenuates synaptic transmission by inducing long-term depression (Teng et al., 2005; Woo et al., 2005), while mature BDNF sustains long-term potentiation and cell survival (Patterson et al., 1996; Lee et al., 2001; Pang et al., 2004). It is therefore conceivable that the biological role of the shift toward higher proBDNF and lower BDNF levels observed after acute stress may be to attenuate proactive behavior and decrease activity in stressed animals. Stress affects the hormonal response in fish in much the same way it does in higher animals. Stress stimulates the hypothalamus, one of the oldest parts of the brain in evolutionary terms, and is responsible for controlling the most basic functions, such as hunger, thirst, and sex drive and, in mammals, body temperature—all functions that are also mediated by BDNF. A reduced behavioral activity may thus represent an adaptive response to dangerous situations, represented here by shallow waters, to immediately save energy and for recovery in preparation for future actions. In this context, it is striking that 100% of animals in the experimental stress group showed a greater than one standard deviation increase in proBDNF levels (and corresponding decrease in mature BDNF).

Mammalian BDNF transcripts produce the well-known 32-kDa propeptide precursor that is cleaved either to pro28kDa or to the mature 14-kDa BDNF forms by two different proteases (Mowla et al., 2001). Pro28kDa BDNF peptide is not further processed into the mature 14-kDa BDNF form, but it represents a true, final proteolytic product generated by a specific Ca^{2+}-dependent serine proteinase known as membrane-bound transcription factor site-1 protease, also known as subtilisin/kexin-isozyme 1 (Seidah et al., 1999); mature 14-kDa BDNF is generated intracellularly by furin (Mowla et al., 2001) or extracellularly by plasmin and matrix metalloprotease-7 (Lee et al., 2001). In contrast, only two BDNF forms are present in sea bass: a proBDNF form corresponding to mammalian pro32KDa precursor

and a mature BDNF. The pro28KDa peptide is absent.

A comparison of *D. labrax* BDNF protein with that of rodents and humans revealed that the mammalian SKI-1 cleavage site at threonine 57 (Arg-Gly-Leu-Thr↓) is absent in fishes and amphibians and first emerged in reptiles during vertebrate evolution (Tettamanti et al., 2010). Limited proteolysis of one inactive precursor to produce active peptides and proteins is a general mechanism to generate biologically diverse products from a single gene. This piece of evidence demonstrates that fishes possess a simplified proteolytic regulation of BDNF and that the pro28KDa proteolytic product, whose function remains to be determined, is absent at this stage of vertebrate evolution.

In conclusion, the proBDNF/totBDNF ratio (or its counterpart matBDNF/totBDNF) is a novel, quantitative, neurological biomarker capable of detecting biological stress in fish. The highly predictable proBDNF/totBDNF ratio for stress in lower vertebrates indicates that processing of BDNF is a central mechanism for adapting to stress and predicts that a similar regulation of pro/mature BDNF has likely been conserved throughout evolution of vertebrates, from fish to humans.

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Chapter 10
The SoLute Carrier (SLC) Family Series in Teleost Fish

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Abstract: Human genes encoding passive transporters, ion-coupled transporters, and exchangers are all included in the so-called SoLute Carrier (SLC) gene series (the Human Genome Organization Gene Nomenclature Committee; http://www.genenames.org/), consisting of 51 families and at least 378 genes (http://www.bioparadigms.org). Ortholog genes encoding for transport proteins of the SLC series have comparatively been described in teleost fish, although their functional properties, in terms of kinetic parameters, substrate specificities, and inhibition patterns of the expressed transport proteins, have only sporadically been assessed in vitro. This chapter gives the latest updates for the SLC families and their members in teleost fish as well as relevant links to GenBank database and literature. By using a functional genomics approach, a list (version 1.0) of all currently known SLC families in teleost fish is provided in the form of SLC tables.

Introduction

The SoLute Carrier (SLC) gene family series encodes membrane transporters (Hediger et al. 2004). In humans, the SLC family series comprises 51 gene families having at least 378 putatively functional protein-coding genes (http://www.bioparadigms.org). The gene products include passive transporters (*alias* facilitated or facilitative transporters or uniporters), cotransporters (*alias* symporters), and exchangers (*alias* antiporters), located in all cellular and subcellular membranes (with the apparent exception of the nuclear membrane). Passive transporters move one molecule at a time down a concentration gradient. Conversely, active transporters couple the movement of one type of ion or molecule against its concentration gradient to the movement of another ion or molecule down its concentration gradient. In particular, a cotransporter moves the transported molecule or ion and the cotransported molecule or ion in the same direction across a membrane, while an exchanger moves the transported molecule or ion and the cotransported molecule or ion in opposite directions. Transported substrates include amino acids and oligopeptides, glucose and other sugars, inorganic cations and anions, bile salts, carboxylate and other organic anions, acetyl coenzyme A, essential metals, biogenic amines, neurotransmitters, vitamins, fatty acids and lipids, nucleosides, ammonium, choline, thyroid hormone and urea, and many other solutes.

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

The SLC Family Series in Teleost Fish

Currently, there are representatives of 50 families in the teleost fish SLC series (the SLC28 family lacks of members so far), with a total of at least 338 putatively functional protein-coding genes (Tables 10.1 and 10.2). A list (version 1.0) of all currently known teleost fish SLC families and their members is provided in the form of SLC tables (Tables 10.3–10.52). Each table provides current (March 2011) relevant information in terms of official gene name, fish species, GenBank database nucleotide accession number (GenBank ID), and literature. Moreover, for each transporter, the predominant substrate(s) is(are) indicated, as it(they) has(have) been assessed in higher vertebrates by using a variety of in vitro heterologous expression systems (see, e.g., Bossi et al., 2007; Markovich, 2008; Musa-Aziz et al., 2010). If a functional analysis has been performed on a transporter cloned from a teleost fish, the (list of) tested substrate(s) is indicated.

Transporters of the SLC series have been cloned from a variety of fish species (Figure 10.1). In our survey, zebrafish (*Danio rerio*) represents the most relevant species with 304 out of 338 genes found, followed by Atlantic salmon (*Salmo salar*) with 53 genes, rainbow trout (*Oncorhynchus mykiss*) with 22, fugu (*Takifugu rubripes*) with 16, mefugu (*Takifugu obscurus*) with 11, and Japanese eel (*Anguilla japonica*) with 10. SLC genes from a variety of fish models popular in genetics, developmental biology studies, and/or genome analysis, among which medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), and spotted green pufferfish (*Tetraodon nigroviridis*), as well as from fish species economically relevant in aquaculture, among which European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*), were less represented in this list, most probably because of incompleteness in annotation process and/or limited submission of sequence information in the public database.

Transport Processes in Teleost Fish via SLC Transporters

The SLC families represent an heterogeneous group of genes, and many structure/function/evolution-based criteria have variably been used for their classification (see, e.g., Hediger et al., 2004; Fredriksson et al., 2008; He et al., 2009; Sreedharan et al., 2010; Hoglund et al., 2011). It is not unusual to find atypical transporters and/or transporters that are represented typically or exclusively in one (or a few) animal group(s). This is the case of teleost fish, which have undergone whole-genome duplication process(es) during the evolution (see, e.g., Ravi and Venkatesh, 2008) and thus often present duplicated SLC genes (see Tables 10.3–10.52). In this paper, the 50 SLC families have been included in 12 groups (Table 10.2), based on the nature of their substrates, in accordance with the description of He et al. (2009). A rapid description of the 12 groups in teleost fish is reported in the following text.

Inorganic Cation/Anion Transport

Eight families constitute the group of proteins that transport exclusively inorganic cations and anions across membranes (Table 10.2; see also Tables 10.6, 10.10, 10.11, 10.14, 10.22, 10.26, 10.28, 10.35, and literature cited therein): slc4, with 11 members found so far in teleost fish, plays a key role in mediating Na^+- and/or Cl^--dependent transport of basic anions (e.g., HCO_3^-, CO_3^{2-}) in various tissues and cell types; slc8, with 7 members, is a group of Na^+/Ca^{2+} exchangers; slc9, with 13 members, comprises Na^+/H^+ exchanger proteins involved in the electroneutral exchange of Na^+ and H^+; slc12, with 13 members, functions as a Na^+, K^+, and Cl^- ion electroneutral symporter; slc34, with 3 members, is an important type II Na^+/HPO_4^{2-} symporter; slc20, with 3 members functions as a type III $Na^+/H_2PO_4^-$ symporter; slc24, with 3 members, is a group of Na^+/Ca^{2+} or Na^+/K^+ exchangers; slc26, with

Table 10.1 List of the currently known SoLute Carrier (SLC) families in teleost fish. The total number of members in each family are shown on the right.

The solute carrier family series in teleost fish	Total
slc1: High-affinity glutamate and neutral amino acid transporter family	13
slc2: Facilitative GLUT transporter family	18
slc3: Heavy subunits of the heteromeric amino acid transporters	2
slc4: Bicarbonate transporter family	11
slc5: Sodium glucose cotransporter family	8
slc6: Sodium- and chloride-dependent neurotransmitter transporter family	16
slc7: Cationic amino acid transporter/ glycoprotein-associated family	8
slc8: $Na^+/Ca2^+$ exchanger family	7
slc9: Na^+/H^+ exchanger family	13
slc10: Sodium bile salt cotransport family	4
slc11: Proton-coupled metal ion transporter family	5
slc12: Electroneutral cation-coupled Cl cotransporter family	13
slc13: Human Na^+–sulfate/carboxylate cotransporter family	5
slc14: Urea transporter family	3
slc15: Proton oligopeptide cotransporter family	3
slc16: Monocarboxylate transporter family	9
slc17: Vesicular glutamate transporter family	7
slc18: Vesicular amine transporter family	4
slc19: Folate/thiamine transporter family	1
slc20: Type III Na^+-phosphate cotransporter family	3
slc21/slco: Organic anion transporter family	14
slc22: Organic cation/anion/zwitterions transporter family	8
slc23: Na^+-dependent ascorbic acid transporter family	2
slc24: $Na^+/(Ca2^+\text{-}K^+)$ exchanger family	3
slc25: Mitochondrial carrier family	51
slc26: Multifunctional anion exchanger family	9
slc27: Fatty acid transporter family	5
slc28: Na^+-coupled nucleoside transport family	0
slc29: Facilitative nucleoside transporter family	1
slc30: Zinc efflux family	8
slc31: Copper transporter family	2
slc32: Vesicular inhibitory amino acid transporter family	1
slc33: Acetyl-CoA transporter family	1
slc34: Type II Na^+–phosphate cotransporter family	3
slc35: Nucleoside-sugar transporter family	22
slc36: Proton-coupled amino acid transporter family	1
slc37: Sugar–phosphate/phosphate exchanger family	3
slc38: System A and system N sodium-coupled neutral amino acid transporter family	8
slc39: Metal ion transporter family	11
slc40: Basolateral iron transporter family	1
slc41: MgtE-like magnesium transporter family	2
slc42: Rh ammonium transporter family	6
slc43: Na^+-independent, system-L-like amino acid transporter family	4
slc44: Choline-like transporter family	5

(Continued)

Table 10.1 (*Continued*)

The solute carrier family series in teleost fish	Total
slc45: Putative sugar transporter family	3
slc46: Folate transporter family	2
slc47: Multidrug and toxin extrusion (mate) family	2
slc48: Heme transporter family	2
slc49: Transporters of the major facilitator superfamily	3
slc50: Sugar efflux transporters	1
slc51: Transporters of steroid-derived molecules	1
Total	*338*

Table 10.2 Classification of teleost fish SLC families based on their substrate specificity.

		Teleost fish members found	Teleost fish members tested for function
Inorganic cation/anion transport			
slc4	Bicarbonate transporter family	11	6
slc8	$Na^+/Ca2^+$ exchanger family	7	2
slc9	Na^+/H^+ exchanger family	13	1
slc12	Electroneutral cation-coupled Cl cotransporter family	13	1
slc20	Type III Na^+–phosphate cotransporter family	3	0
slc24	$Na^+/(Ca^{2+}\text{-}K^+)$ exchanger family	3	0
slc26	Multifunctional anion exchanger family	9	3
slc34	Type II Na^+–phosphate cotransporter family	3	2
Total		*62*	*15*
Amino acid and oligopeptide transport			
slc1	High-affinity glutamate and neutral amino acid transporter family	13	0
slc3	Heavy subunits of the heteromeric amino acid transporters	2	0
slc7	Cationic amino acid transporter/ glycoprotein-associated family	8	0
slc15	Proton oligopeptide cotransporter family	3	2
slc17	Vesicular glutamate transporter family	7	0
slc32	Vesicular inhibitory amino acid transporter family	1	0
slc36	Proton-coupled amino acid transporter family	1	0
slc38	System A and System N sodium-coupled neutral amino acid transporter family	8	0
slc43	Na^+-independent, system-L-like amino acid transporter family	4	0
Total		*47*	*2*

Table 10.2 (*Continued*)

		Teleost fish members found	Teleost fish members tested for function
Transport of glucose and other sugars			
slc2	Facilitative GLUT transporter family	18	4
slc5	Sodium glucose cotransporter family	8	2
slc37	Sugar–phosphate/phosphate exchanger family	3	0
slc45	Putative sugar transporter family	3	0
slc50	Sugar efflux transporters	1	0
Total		*33*	*6*
Transport of bile salts and organic anions			
slc10	Sodium bile salt cotransport family	4	0
slc13	Human Na^+–sulfate/carboxylate cotransporter family	5	2
slc16	Monocarboxylate transporter family	9	0
slc47	Multidrug and toxin extrusion (mate) family	2	0
slc51	Transporters of steroid-derived molecules	1	0
Total		*21*	*2*
Metal ion transport			
slc11	Proton-coupled metal ion transporter family	5	2
slc30	Zinc efflux family	8	1
slc31	Copper transporter family	2	0
slc39	Metal ion transporter family	11	2
slc40	Basolateral iron transporter family	1	1
slc41	MgtE-like magnesium transporter family	2	0
Total		*29*	*6*
Transport of urea, neurotransmitters and biogenic amines, ammonium and choline			
slc6	Sodium- and chloride-dependent neurotransmitter transporter family	16	3
slc14	Urea transporter family	3	2
slc18	Vesicular amine transporter family	4	0
slc22	Organic cation/anion/zwitterion transporter family	8	1
slc42	Rh ammonium transporter family	6	4
slc44	Choline-like transporter family	5	0
Total		*42*	*10*
Transport of vitamins and cofactors			
slc19	Folate/thiamine transporter family	1	0
slc23	Na^+-dependent ascorbic acid transporter family	2	0
slc33	Acetyl-CoA transporter family	1	0
slc46	Folate transporter family	2	0
Total		*6*	*0*
Nucleoside/nucleotide transport			
slc28	Na^+-coupled nucleoside transport family	0	0
slc29	Facilitative nucleoside transporter family	1	0
slc35	Nucleoside-sugar transporter family	22	0
Total		*23*	*0*

(Continued)

Table 10.2 (*Continued*)

		Teleost fish members found	Teleost fish members tested for function
Transport of fatty acids, prostaglandins, and steroid sulphates			
slc27	Fatty acid transporter family	5	0
Total		*5*	*0*
Transport of organic anions, prostaglandins and steroid sulfates, thyroid hormones			
slc21/slco	Organic anion transporter family	14	0
Total		*14*	*0*
Heme transport			
slc48	Heme transporter family	2	0
slc49	Transporters of the major facilitator superfamily	3	0
Total		*5*	*0*
Transport across mitochondrial membranes			
slc25	Mitochondrial carrier family	51	0
Total		*51*	*0*

9 members, is a transepithelial multifunctional anion (e.g., sulfate, oxalate, HCO_3^-, Cl^-) exchanger family.

Amino Acid and Oligopeptide Transport

Nine families are involved as transporters of amino acids and/or oligopeptides (Table 10.2; see also Tables 10.3, 10.5, 10.9, 10.17, 10.19, 10.33, 10.37, 10.39, and 10.44, and literature cited therein): slc1, with 13 members found so far in teleost fish, transports high-affinity glutamate and neutral amino acids; slc3, with 2 members, encodes the heavy subunits of the heteromeric amino acid transporters; slc7, with 8 members, represents a group of cationic amino acid/glycoprotein-associated transporters; slc15, with 3 members, represents a family of H^+-oligopeptide symporters; slc17, with 7 members, is involved in various processes ranging from the vesicular storage of the glutamate to the degradation and metabolism of glycoproteins; slc32, with 1 member, transports amino acids across vesicle membranes; slc36, with 1 member, is involved in H^+-coupled amino acid transport; slc38, with 8 members, functions as a Na^+-coupled neutral amino acid transporter; slc43, with 4 members, represents the Na^+-independent system-L-like (that mediates the movement of bulky neutral amino acids across cell membranes) amino acid transporter family. slc16 and slc22 also transport amino acids (refer to following the text).

Transport of Glucose and Other Sugars

Five SLC families function as sugar transporters (Table 10.2; see also Tables 10.4, 10.7, 10.38, 10.46, 10.51, and literature cited therein): slc2, with 18 members found so far in teleost fish, is the facilitative glucose transporter (GLUT) family; slc5, with 8 members, functions as a Na^+/glucose symporter; slc37, with 3 members, is a group of sugar-exchangers; slc45, with 3 members, appears to function as a H^+/sugar symporter. slc50 seems to be a facilitative glucose transporter.

Table 10.3 The solute carrier family 1 (the high-affinity glutamate and neutral amino acid transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc1a1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	Excitatory amino acid transporter 3	*Danio rerio*	NM_001002666.1	Rico et al., 2010; Gesemann et al., 2010a, 2010b	L-Glu,[b] D/L-Asp[b]
slc1a2a	Solute carrier family 1 (glial high affinity glutamate transporter), member 2a	EAAT2b; SLC1A2b; excitatory amino acid transporter SLC1A2b	*D. rerio*	NM_001190305.1	Gesemann et al., 2010a, 2010b	L-Glu,[b] D/L-Asp[b]
slc1a2b	Solute carrier family 1 (glial high affinity glutamate transporter), member 2b	Excitatory amino acid transporter 2; solute carrier family 1 (glial high affinity glutamate transporter), member 2	*D. rerio*	NM_199979.1	Rohrschneider et al., 2007; Toro et al., 2009; Gesemann et al. 2010b; Rico et al., 2010	L-Glu,[b] D/L-Asp[b]
slc1a3a	Solute carrier family 1 (glial high affinity glutamate transporter), member 3a	EAAT1A; excitatory amino acid transporter SLC1A3a; glasta; solute carrier family 1 (glial high affinity glutamate transporter), member 3	*D. rerio*	NM_212640.1	Bae et al., 2009; Gesemann et al., 2010a, 2010b; Rico et al., 2010; Mueller et al., 2010	L-Glu,[b] D/L-Asp[b]
slc1a3b	Solute carrier family 1 (glial high affinity glutamate transporter), member 3b	EAAT1B; excitatory amino acid transporter SLC1A3b; glastb	*D. rerio*	NM_001190303.1	Bae et al., 2009; Gesemann et al., 2010a, 2010b; Rico et al., 2010	L-Glu,[b] D/L-Asp[b]
slc1a4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Neutral amino acid transporter A	*D. rerio*	NM_001002513.2	Gesemann et al., 2010a, 2010b	L-Ala,[c] L-Ser,[c] L-Cys[c]
slc1a5	Solute carrier family 1 (neutral amino acid transporter), member 5	Neutral amino acid transporter B(0)	*D. rerio*	NM_001190755.1	Steffen et al., 2007; Gesemann et al., 2010a, 2010b	L-Ala,[c] L-Ser,[c] L-Thr,[c] L-Cys,[c] L-Gln[c]

(*Continued*)

Table 10.3 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc1a6	Solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6	EAAT1C; EAAT4; excitatory amino acid transporter 4; excitatory amino acid transporter SLC1A6, glastc	*D. rerio*	NM_001109703.1	Bae et al., 2009; Gesemann et al., 2010a, 2010b; Rico et al., 2010	L-Glu,[b] D/L-Asp[b]
slc1a7a	Solute carrier family 1 (glutamate transporter), member 7a	EAAT5A; excitatory amino acid transporter 5; excitatory amino acid transporter SLC1A7a; solute carrier family 1 (glutamate transporter), member 7	*D. rerio*	NM_001130622.1	Gesemann et al., 2010a, 2010b; Rico et al., 2010	L-Glu,[b] D/L-Asp[b]
slc1a7b	Solute carrier family 1 (glutamate transporter), member 7b	EAAT5B; excitatory amino acid transporter SLC1A7b	*D. rerio*	NM_001190760.1	Gesemann et al., 2010a, 2010b	L-Glu,[b] D/L-Asp[b]
slc1a8a	Solute carrier family 1 (glutamate transporter), member 8a	Excitatory amino acid transporter SLC1A8a	*D. rerio*	HM138700.1	Gesemann et al., 2010a, 2010b	
slc1a8b	Solute carrier family 1 (glutamate transporter), member 8b	EAAT5B; EAAT6b; excitatory amino acid transporter SLC1A8b; solute carrier family 1 (glutamate transporter), member 7	*D. rerio*	NM_001190816.1	Gesemann et al., 2010a, 2010b	
slc1a9	Solute carrier family 1 (glutamate transporter), member 9	EAAT7; excitatory amino acid transporter SLC1A9	*D. rerio*	NM_001190759.1	Gesemann et al., 2010a, 2010b	

[a] From www.bioparadigms.org.
[b] Na^+-coupled, H^+-coupled, K^+-coupled cotransport.
[c] Na^+-coupled cotransport, amino acid exchange.

Table 10.4 The solute carrier family 2 (the facilitative GLUT transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc2a1a	Solute carrier family 2 (facilitated glucose transporter), member 1a	Facilitated glucose transporter 1; solute carrier family 2, facilitated glucose transporter member 1; solute carrier family 2, member 1; zglut1b	*Danio rerio*	NM_001039808.1	Jensen et al., 2006; ; Tseng et al., 2009b; van Rooijen et al., 2009; Cruz et al., 2010; Hyvärinen et al., 2010; Jensen et al., 2010; Zheng et al., 2009, 2010b		
slc2a1b	Solute carrier family 2 (facilitated glucose transporter), member 1b	Glut1a	*D. rerio* *Oncorhynchus mykiss* *Dicentrarchus labrax* *Cyprinus carpio* *Oreochromis niloticus* *Gadus morhua*	XM_002662528.1 AF247728.1 FQ310506.1 AF247730.1 FJ914657.1 AY526497.1	Cruz et al., 2010; Zheng et al., 2010b; Tseng et al., 2011 Teerijoki et al., 2000; Teerijoki et al., 2001; Hrytsenko et al., 2010; Hall et al., 2004	Glucose,[b] galactose,[b] mannose,[b] glucosamine[b]	Glucose[c,d]
slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2	Solute carrier family 2, facilitated glucose transporter member 2; glut2	*D. rerio* *G. morhua* *Osmerus mordax* *D. labrax* *O. mykiss*	NM_001042721.1 AY795481.1 FJ797642.1 EF014277.2 NM_001124289.1	Castillo et al., 2009; Ngan and Wang, 2009; Tseng et al., 2009b; Hall et al., 2006; Terova et al., 2009b; Krasnov et al., 2001	Glucose,[b] galactose,[b] fructose,[b] mannose,[b] glucosamine[b]	
slc2a3	Solute carrier family 2 (facilitated glucose transporter), member 3	Solute carrier family 2, facilitated glucose transporter member 3; glut3	*D. rerio* *G. morhua* *Salmo salar*	NM_001002643.1 AY645944.1 BT059743.1	Tseng et al., 2009b; van Rooijen et al., 2009; Zheng et al., 2010b; Hall et al., 2005	Glucose,[b] galactose,[b] mannose,[b] xylose[b]	
LOC100330897	Solute carrier family 2, facilitated glucose transporter member 3-likes		*D. rerio* *Ctenopharyngodon idella*	XM_002667123.1 AY231476.1	Zhang et al., 2003		

(Continued)

Table 10.4 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc2a4	Solute carrier family 2, member 4	Glucose transporter 4	*G. morhua* *Oncorhynchus kisutch* *Salmo trutta*	DQ109810.1 AF502957.1 AAG12191.1	Hall et al., 2006; Capilla et al., 2004; Planas et al., 2000	Glucose,[b] glucosamine[b]	2-Deoxyglucose[e]
slc2a5	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5		*D. rerio*	NR_023322.1	Tseng et al., 2009b	Fructose[b]	
LOC560602	Novel protein similar to vertebrate solute carrier family 2 (facilitated glucose transporter), member 6 (SLC2A6)	Solute carrier family 2 (facilitated glucose transporter), member 6	*D. rerio*	XM_684000.5	Tseng et al., 2009b; Tseng et al., 2011	Glucose	Glucose[c]
slc2a8	Solute carrier family 2 (facilitated glucose transporter), member 8	Solute carrier family 2, facilitated glucose transporter member 8; zglut8	*D. rerio*	NM_212798.1	Tseng et al., 2009b; Cruz et al., 2010	Glucose,[b] fructose,[b] galactose[b]	
slc2a9l1	Solute carrier family 2 (facilitated glucose transporter), member 9-like 1	zglut9a	*D. rerio*	NM_001166117.1	Tseng et al., 2009b	Urate (glucose, fructose)	
slc2a9l2	Solute carrier family 2 (facilitated glucose transporter), member 9-like 2	zglut9c	*D. rerio*	XM_001918919.4	Tseng et al., 2009b	Urate (glucose, fructose)	
slc2a10	Solute carrier family 2 (facilitated glucose transporter), member 10	Solute carrier family 2, facilitated glucose transporter member 10; zglut10	*D. rerio*	NM_001111163.1	Tseng et al., 2009b	Glucose,[b] galactose[b]	

slc2a11b	Solute carrier family 2 (facilitated glucose transporter), member 11b	fc06b08; zglut11b	*D. rerio*	NM_001114430.1	Leung et al., 2007; Tseng et al., 2009b	Glucose,[b] fructose[b]	
slc2a11l	Solute carrier family 2 (facilitated glucose transporter), member 11-like	zglut11c	*D. rerio*	NM_001080016.1	Tseng et al., 2009b	Glucose,[b] fructose[b]	
slc2a12	Solute carrier family 2 (facilitated glucose transporter), member 12	GLUT-12; glucose transporter type 12; solute carrier family 2, facilitated glucose transporter member 12; zglut12	*D. rerio*	NM_200538.1	Tseng et al., 2009b	Glucose[b]	
slc2a13	Solute carrier family 2 (facilitated glucose transporter), member 13	Glucose transporter 13a; proton myo-inositol cotransporter; solute carrier family 2 (facilitated glucose transporter), member 13a; zglut13.1; zglut13.2	*D. rerio*	NM_001159829.1	Tseng et al., 2009b; Cruz et al., 2010; Tseng et al., 2011	Myo-inositol[f]	Glucose[c]
slc2a15a	Solute carrier family 2 (facilitated glucose transporter), member 15a	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	*D. rerio*	NM_001162541.1	Wotton et al., 2008; Komisarczuk et al., 2009		
slc2a15b	Solute carrier family 2 (facilitated glucose transporter), member 15b	zglut9b	*D. rerio*	NM_001020494.1	Leung et al., 2007; Wotton et al., 2008; Tseng et al., 2009b	Urate (glucose, fructose)	

[a] From www.bioparadigms.org.
[b] Facilitated transport.
[c] Zebrafish.
[d] Rainbow trout.
[e] Coho salmon.
[f] H^+-coupled transport.

Table 10.5 The solute carrier family 3 (the heavy subunits of the heteromeric amino acid transporters). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc3a1	Solute carrier family 3, member 1	Neutral and basic amino acid transport protein rBAT	*Salmo salar*	NM_001173963.1		See details in slc7 table
slc3a2	Solute carrier family 3, member 2	4f2hc	*Danio rerio* *S. salar*	NM_131601.1 BT045623.1 BT059429.1	Yoder and Litman, 2000; Hart et al., 2007	See details in slc7 table

[a] From www.bioparadigms.org.

Transport of Bile Salts and Organic Anions

Five families participate as transporters of bile salts, organic anions, and bile conjugates (Table 10.2; see also Tables 10.12, 10.15, 10.18, 10.48, 10.52, and literature cited therein): slc10, with 4 members found so far in teleost fish, is involved in bile acid transport; slc13, with 5 members, is the Na^+/sulphate/selenate/thiosulphate/carboxylate symporter family (the transported di- and tricarboxylates include succinate, citrate, and α-ketoglutarate); slc16, with 9 members, is involved in the H^+-linked transport of monocarboxylate anions (e.g., lactate, pyruvate and ketone bodies) and aromatic amino acids; slc47, with 2 members, has been characterized as a polyspecific H^+/organic cation exporter family. The slc47 genes are also known as multidrug and toxicant extrusion 1 (mate1) and 2 (mate2) genes. slc51, with 1 member, participates in the transport of glycine and taurine conjugated bile acid species. slcotype subfamilies also participate in organic anion transport (refer to following the text).

Metal Ion Transport

Six SLC families are involved in metal ion transport (Table 10.2; see also Tables 10.13, 10.31, 10.32, 10.40, 10.41, 10.42, and literature cited therein): slc11, with 5 members found so far in teleost fish, groups H^+-coupled metal ion influx transporters, also known as the natural resistance-associated macrophage protein (nramp) homologues; slc30, with 8 members, is involved in Zn^{2+} efflux; slc31, with 2 members, is a copper influx transporter family; slc39, with 11 members, functions in the influx of essential metals such as Zn^{2+}, Fe^{2+}, Cu^{2+}, and Mn^{2+}, although nonessential toxic metals such as Cd^{2+}, Pb^{2+}, and Hg^{2+} can compete with at least two of these transporters; slc40, with 1 member, is a basolateral iron transporter; slc41, with 2 members, is the MgtE-like magnesium transporter family, whose physiological role in eukaryotes remains unclear.

Transport of Urea, Neurotransmitters and Biogenic Amines, Ammonium, and Choline

Six families participate in the transport of these molecules (Table 10.2; see also Tables 10.8, 10.16, 10.20, 10.24, 10.43, 10.45, and literature cited therein): slc6, with 16 members so far found in teleost fish, represents Na^+ and Cl^- ion-dependent neurotransmitter (γ-aminobutyric acid, serotonin, dopamine, and norepinephrine) transporters;

Table 10.6 The solute carrier family 4 (the bicarbonate cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc4a1a	Solute carrier family 4, anion exchanger, member 1a	AE1/band 3; erythroid anion exchanger 1; ret; retsina; solute carrier family 4, anion exchanger, member 1; AE1a	*Danio rerio* *Oncorhynchus mykiss* *Oreochromis mossambicus* *Zoarces viviparus* *Salmo salar*	NM_198338.1 NM_001124717.1 AY322479.1 EU552533.1 BT072321.1	Hübner et al., 1992; Paw, 2001; North and Zon, 2003; Paw et al., 2003; Traver et al., 2003; Davidson and Zon, 2004; Guizouarn et al., 2005; Juarez et al., 2005; Qian et al., 2005; Liu et al., 2007a; Nunomura et al., 2007; Deigweiher et al., 2008; Sidi et al., 2008; Sundström et al., 2008; Amigo et al., 2009; Belele et al., 2009; Kitaguchi et al., 2009; Nilsson et al., 2009; Schwarz et al., 2009; Sun et al., 2009; Lee et al., 2011	Cl^-, HCO_3^{-b}	$Cl^{-,c,d}$ taurine[d]
slc4a1b	Solute carrier family 4, anion exchanger, member 1b	Solute carrier family 4 anion exchanger member 1b; AE1b	*D. rerio* *Dicentrarchus labrax*	NM_001168266.1 FQ310507.1	Hwang, 2009; Lee et al., 2011		
slc4a2a	Solute carrier family 4, anion exchanger, member 2a	ae2; ae2.1; anion exchanger 2; solute carrier family 4, anion exchanger, member 2	*D. rerio*	NM_001037237.1	Shmukler et al., 2005; Ma and Jiang, 2007; Wingert et al., 2007; Mudumana et al., 2008; Shmukler et al., 2008; Sundström et al., 2008; Fauny et al., 2009; Lee et al., 2011	$Cl^{-,b}$ HCO_3^{-b}	$Cl^{-,c}$ HCO_3^{-c}
slc4a2b	Solute carrier family 4, anion exchanger, member 2b	ae2.2; anion exchanger 2.1	*D. rerio*	NM_001114440.1	Shmukler et al., 2008; Lee et al., 2011	$Cl^{-,b}$ HCO_3^{-b}	$Cl^{-,c}$ HCO_3^{-c}

(Continued)

Table 10.6 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc4a4a	Solute carrier family 4, member 4a	NBC1; electrogenic Na^+ bicarbonate cotransporter; electrogenic sodium bicarbonate cotransporter 1; solute carrier family 4, member 4	*D. rerio* *Fundulus heteroclitus* *Opsanus beta*	NM_001034984.1 GQ376030.1 FJ463158.1	Nichane et al., 2006; Wingert et al., 2007; Mudumana et al., 2008; Sussman et al., 2009; Vasilyev et al., 2009; de Groh et al., 2010; Lee et al., 2011 Taylor et al., 2010	HCO_3^- (and/or CO_3^{2-})[e]	HCO_3^-,[c] Na^+[c,f]
slc4a4b	Solute carrier family 4, sodium bicarbonate cotransporter, member 4b	Sodium bicarbonate cotransporter; solute carrier family 4, member 4	*D. rerio* *Tribolodon hakonensis*	XM_685028.5 AB055467.1	Yan et al., 2007; Lee et al., 2011; Hirata et al., 2003	HCO_3^- (and/or CO_3^{2-})[e]	HCO_3^-,[g] Na^+[g]
slc4a4	Solute carrier family 4, sodium bicarbonate cotransporter, member 4		*Takifugu obscurus* *Z. viviparus* *O. mossambicus* *O. mykiss*	AB362567.1 EU552532.1 AB562944.1 NM_001124325.1	Kurita et al., 2008; Deigweiher et al., 2008; Furukawa et al., 2011	HCO_3^- (and/or CO_3^{2-})[e]	HCO_3^-[h]
slc4a5	Solute carrier family 4, sodium bicarbonate cotransporter, member 5		*D. rerio*	XM_001921788.1		HCO_3^- (and/or CO_3^{2-})[e]	

LOC559268	Novel protein similar to vertebrate solute carrier family 4, sodium bicarbonate cotransporter, member 5 (SLC4A5)	Sodium bicarbonate transporter 4	*D. rerio*	XM_682596.5		
slc4a8	Solute carrier family 4, sodium bicarbonate cotransporter, member 8		*D. rerio*	XM_680574.4		Cl^{-},[b] HCO_3^{-}[b]
slc4a11	Solute carrier family 4, sodium borate transporter, member 11	Sodium bicarbonate transporter-like protein 11; solute carrier family 4 member 11	*D. rerio*	NM_001159828.1	Lee et al., 2011	borate ($B(OH)_4^{-}$),[e] Na^{+},[e] OH^{-}[e]

[a] From www.bioparadigms.org.
[b] Cl^{-}/HCO_3^{-} exchange.
[c] Zebrafish.
[d] Rainbow trout.
[e] Na^{+}-coupled transport.
[f] Gulf toadfish.
[g] Osorezan dace.
[h] Mefugu.

Table 10.7 The solute carrier family 5 (the sodium glucose cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc5a1	Solute carrier family 5 (sodium/glucose cotransporter), member 1	SGLT1; sodium/glucose cotransporter 1	*Danio rerio* *Salmo salar*	NM_200681.1 NM_001171787.1	Wingert et al., 2007; Hegedus et al., 2009	Glucose,[b] galactose[b]	
slc5a2	Solute carrier family 5 (sodium/glucose cotransporter), member 2	Sodium/glucose cotransporter 2	*D. rerio* *Oncorhynchus mykiss*	NM_212926.1 NM_001124432.1		Glucose[c]	
slc5a3	Solute carrier family 5 (inositol transporters), member 3		*D. rerio*	NM_001159837.1		Myo-inositol[c]	
slc5a5	Solute carrier family 5 (sodium iodide symporter), member 5	Sodium/iodide cotransporter	*D. rerio*	NM_001089391.1	Alt et al., 2006; Wendl et al., 2007	I^{-}[c] (ClO_4^{-},[c] SCN^{-},[c] NO_3^{-},[c] Br^{-}[c])	
slc5a8l	Solute carrier family 5 (iodide transporter), member 8-like	Electrogenic sodium monocarboxylate cotransporter; sodium solute symporter family 5 member 8 protein; sodium-coupled monocarboxylate transporter 1; solute carrier family 5 member 8; zSMCTe	*D. rerio*	NM_001034969.1	Plata et al., 2007	Short-chain fatty acids,[c] lactate,[c] nicotinate[c]	Lactate,[d] nicotinate,[d] pyruvate,[d] butyrate,[d] virtually all monocarboxylates[b]

slc5a9	Solute carrier family 5 (sodium/glucose cotransporter), member 9	Na(+)/glucose cotransporter 4; sodium/glucose cotransporter 4; solute carrier family 5 member 9	*D. rerio*	NM_001113605.1		Mannose[c]	
slc5a11	Solute carrier family 5 (sodium/glucose cotransporter), member 11	Sodium/myo-inositol cotransporter 2	*D. rerio*	NM_001007300.1		Myo-inositol,[c] Chiro-inositol[c]	
slc5a12	Solute carrier family 5 (sodium/glucose cotransporter), member 12	Electroneutral sodium monocarboxylate cotransporter; low-affinity sodium-lactate cotransporter; sodium-coupled monocarboxylate transporter 2; solute carrier family 5 member 12; zSLC5A12; zSMCTn	*D. rerio*	NM_200368.1	Plata et al., 2007	Short-chain fatty acids,[c] lactate,[c] pyruvate,[c] nicotinate[c]	Lactate,[d] nicotinate,[d] pyruvate,[d] butyrate,[d] virtually all monocar-boxylates[b]

[a]From www.bioparadigms.org.
[b]$Na^+(H^+)$-coupled cotransport.
[c]$Na^+(H^+)$-coupled cotransport.
[d]Zebrafish.

Table 10.8 The solute carrier family 6 (the sodium- and chloride-dependent neurotransmitter transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
zgc:103663	zgc:103663	Sodium- and chloride-dependent GABA transporter 1; solute carrier family 6 (neurotransmitter transporter, GABA), member 1	*Danio rerio*	NM_001007362.1	Yang et al., 2007	GABA[b]	
slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Dopamine transporter; sodium-dependent dopamine transporter	*D. rerio*	NM_131755.1	Holzschuh et al., 2001; Holzschuh et al., 2003; Lam et al., 2005; McKinley et al., 2005; Jeong et al., 2006; Prober et al., 2006; Wang et al., 2006; Blechman et al., 2007; Filippi et al., 2007; Hart et al., 2007; Ryu et al., 2007; Shang and Zhdanova, 2007; Yokogawa et al., 2007; López Patiño et al., 2008; Luo et al., 2008; Russek-Blum et al., 2008; Bai and Burton, 2009; Boehmler et al., 2009; Foley et al., 2009; Filby et al., 2010; Filippi et al., 2010; Li et al., 2010; ; Sallinen et al., 2010; Sheng et al., 2010; Yamamoto et al., 2010; Yamamoto et al., 2011;	Dopamine[b]	
slc6a4a	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4A	Serotonin transporter a; serta; sodium-dependent serotonin transporter	*D. rerio*	NM_001039972.1	Wang et al., 2006; Airhart et al., 2007; Norton et al., 2008; Severinsen et al., 2008; Filby et al., 2010; Oehlers et al., 2011	Serotonin[c]	Serotonin[d]
slc6a4b	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4B	Serotonin transporter b; sertb	*D. rerio*	NM_001177459.1	Wang et al., 2006; Filby et al., 2010; Oehlers et al., 2011	Serotonin[c]	

slc6a5	Solute carrier family 6 (neurotransmitter transporter, glycine), member 5	Glycine transporter 2; sodium- and chloride-dependent glycine transporter 2	*D. rerio*	NM_001009557.1	Higashijima et al., 2004a, 2004b; Cui et al., 2005; McLean et al., 2007; Yokogawa et al., 2007; Batista and Lewis, 2008; Batista et al., 2008; Desai et al., 2008; Mongeon et al., 2008; Satou et al., 2009; Webb et al., 2009	Glycine[b]	
slc6a6	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	fc26e12; solute carrier family 6, member 6; taurine transporter	*D. rerio* *Salmo salar* *Oreochromis mossambicus*	NM_001037661.1 NM_001123630.1 AB033497.1	Kozlowski et al., 2008 Takeuchi et al., 2000	Taurine,[b] beta-alanine[b]	Taurine[d]
zgc:158225	zgc:158225	Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	*D. rerio*	NM_001080580.1		L-proline[b]	
slc6a8	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	Sodium- and chloride-dependent creatine transporter 1; ct1	*D. rerio*	XM_690840.2	Wang et al., 2007, 2010, Nagayoshi et al., 2008	Creatine[b]	
slc6a9	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9	Sho; shocked; sodium- and chloride-dependent glycine transporter 1; solute carrier family 6 member 9	*D. rerio*	NM_001030073.1	Higashijima et al., 2004a; Cui et al., 2005; Mongeon et al., 2008	Glycine[b]	Glycine[d]
slc6a11	Solute carrier family 6 (neurotransmitter transporter, GABA), member 11		*D. rerio*	NM_001098387.1	Liu et al., 2003	GABA[b]	
slc6a13	Solute carrier family 6 (neurotransmitter transporter, GABA), member 13		*D. rerio* *S. salar*	NM_001004533.1 BT059203.1		GABA[b]	

(Continued)

Table 10.8 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc6a13l	Solute carrier family 6 (neurotransmitter transporter, GABA), member 13 like		*D. rerio*	NM_001122761.1			
slc6a17	Solute carrier family 6, member 17		*D. rerio*	XM_001335964.2		Neutral amino acids[e]	
slc6a18	Solute carrier family 6, member 18	Sodium-dependent neutral amino acid transporter B(0)AT3	*D. rerio*	NM_001114897.1		Glycine	
slc6a19a	Solute carrier family 6 (neurotransmitter transporter), member 19a		*D. rerio* *S. salar*	NM_001098178.1 BT046008.1		Neutral amino acids[e]	
slc6a19b	Solute carrier family 6 (neurotransmitter transporter), member 19b	Sodium-dependent neutral amino acid transporter B(0)AT1; solute carrier family 6 (neurotransmitter transporter), member 19	*D. rerio*	NM_199736.1		Neutral amino acids[e]	

[a] From www.bioparadigms.org.
[b] Na^+-coupled, Cl^--coupled cotransport.
[c] Na^+-coupled, Cl^--coupled, K^+-coupled cotransport.
[d] Zebrafish.
[e] Na^+-coupled cotransport.

Table 10.9 The solute carrier family 7 (the cationic amino acid transporter/glycoprotein-associated family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc7a2	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 2	CAT-2; CAT2; low affinity cationic amino acid transporter 2; solute carrier family 7 member 2	*Danio rerio*	NM_001008584.1		Cationic L-amino acids[b]
slc7a3	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 3	Solute carrier family 7, member 3	*D. rerio*	NM_001007329.2		Cationic L-amino acids[b]
slc7a4	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 4	Cationic amino acid transporter 4	*D. rerio*	NM_194427.2		
slc7a6	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 6	y^+ L-amino acid transporter 2; solute carrier family 7 member 6; y(+)L-type amino acid transporter 2; y^+LAT-2; y^+LAT2; zfSlc7a6	*D. rerio*	NM_001020474.1	Kawakami et al., 2005	Na^+-independent: cationic amino acids[c]; Na^+-dependent: large neutral amino acids[c]
slc7a7	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 7	y^+L-amino acid transporter 1	*D. rerio* *Salmo salar*	NM_001037559.1 BT045116.1		Na^+-independent: cationic amino acids[c] Na^+-dependent: large neutral amino acids[c]
slc7a8	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 8		*D. rerio*	NM_001007184.1		Neutral L-amino acids,[c] BCH[c]
slc7a9	Carrier family 7 (cationic amino acid transporter, y^+ system), member 9		*D. rerio* *S. salar*	XM_690408.2 BT059579.1		Cationic amino acids,[c] large neutral amino acids[c]
slc7a10	Solute carrier family 7, (neutral amino acid transporter, y^+ system) member 10		*D. rerio*			Small neutral amino acids[c]

[a] From www.bioparadigms.org.
[b] Facilitated transport.
[c] Amino acid exchange.

Table 10.10 The solute carrier family 8 (the Na^+/Ca^{2+} exchanger family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc8a1a	Solute carrier family 8 (sodium/calcium exchanger), member 1a	Cardiac-specific sodium-calcium exchanger 1; ncx1a; sodium calcium exchanger 1h; tre; tremblor	*Danio rerio*	NM_001037102.1	Chen et al., 1996; Ebert et al., 2005; Langenbacher et al., 2005; Marshall et al., 2005; Wenzlau et al., 2006; Liao et al., 2007; Ebert et al., 2008; Leung et al., 2008	Na^+, Ca^{2+}[b]	Ca^{2+}[c]
slc8a1b	Solute carrier family 8 (sodium/calcium exchanger), member 1b	ncx1n; sodium calcium exchanger 1n; sodium/calcium exchanger 1	*D. rerio*	NM_001039144.1	Langenbacher et al., 2005; Liao et al., 2007; Tseng et al., 2009a; Janicke et al., 2010	Na^+, Ca^{2+}[b]	
slc8a2a	Solute carrier family 8 (sodium-calcium exchanger), member 2a	ncx2a; sodium/calcium exchanger 2; solute carrier family 8 (sodium/calcium exchanger), member 2	*D. rerio*	NM_001123296.1	Liao et al., 2007	Na^+, Ca^{2+}[b]	
slc8a2b	Solute carrier family 8 (sodium-calcium exchanger), member 2b	ncx2b; sodium/calcium exchanger 2	*D. rerio*	NM_001123284.1	Liao et al., 2007	Na^+, Ca^{2+}[b]	
slc8a3	Solute carrier family 8 (sodium-calcium exchanger), member 3	ncx3; sodium/calcium exchanger 3	*D. rerio*	NM_001123256.1	Marshall et al., 2005; Liao et al., 2007; Shu et al., 2007	Na^+, Ca^{2+}[b]	
slc8a4a	Solute carrier family 8 (sodium/calcium exchanger), member 4a	ncx4a; sodium/calcium exchanger 4	*D. rerio*	NM_001089419.1	Marshall et al., 2005; Liao et al., 2007; Shu et al., 2007; On et al., 2009	Na^+, Ca^{2+}[b]	Na^+, Ca^{2+}[c]
slc8a4b	Solute carrier family 8 (sodium/calcium exchanger), member 4b	Sodium/calcium exchanger 4	*D. rerio*	NM_001123240.1	Marshall et al., 2005; Liao et al., 2007; Schonthaler et al., 2010	Na^+, Ca^{2+}[b]	

[a] From www.bioparadigms.org.
[b] Na^+/Ca^{2+} exchange.
[c] Zebrafish.

Table 10.11 The solute carrier family 9 (the Na^+/H^+ exchanger family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc9a1	Solute carrier family 9 (sodium/hydrogen exchanger), member 1	Sodium hydrogen exchanger 1; sodium/hydrogen exchanger 1	*Danio rerio* *Oncorhynchus mykiss* *Takifugu obscurus* *Pseudopleuronectes americanus* *Platichthys flesus* *Cyprinus carpio* *Anguilla anguilla*	NM_001113480.1 NM_001124695.1 AB200332.1 AY174870.1 AJ006918.1 AJ006916.1 AJ006917.1	Yan et al., 2007; Borgese et al., 1992; Parks et al., 2010; Kato et al., 2005; Pedersen et al., 2003	Na^+,[b] H^+,[b] Li^+,[b] NH_4^+[b]	Na^+,[c,d] H^+[c,d]
slc9a2	Solute carrier family 9 (sodium/hydrogen exchanger), member 2	Sodium hydrogen exchanger 2; sodium/hydrogen exchanger 2	*D. rerio* *O. mykiss* *Myoxocephalus octodecemspinosus* *Tetraodon nigroviridis*	NM_001114095.1 NM_001130994.1 AF159879.2 CAAE01014781.1	Craig et al., 2007; Yan et al., 2007; Ivanis et al., 2008; Claiborne et al., 1999	Na^+,[b] H^+,[b] Li^+,[b] NH_4^+[b]	
nhe2a	Na+/H+ exchanger isoform 2 subtype a		*Fundulus heteroclitus*	AY818824.1	Scott et al., 2005	Na^+,[b] H^+,[b] Li^+,[b] NH_4^+[b]	
nhe3	Sodium/hydrogen exchanger isoform 3		*M. octodecemspinosus* *Oreochromis mossambicus*	EU909191.1 AB326212.1	Watanabe et al., 2008	Na^+,[b] H^+,[b] Li^+,[b] NH_4^+[b]	

(Continued)

Table 10.11 *(Continued)*

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
nhe3(a)	Sodium/hydrogen exchanger isoform 3		*O. mykiss*	NM_001130995.1	Ivanis et al., 2008	$Na^{+,b}$ $H^{+,b}$ $Li^{+,b}$ NH_4^{+b}	
nhe3(b)	Sodium/hydrogen exchanger isoform 3		*O. mykiss*	NM_001160482.1	Ivanis et al., 2008	$Na^{+,b}$ $H^{+,b}$ $Li^{+,b}$ NH_4^{+b}	
slc9a3.1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3.1	Sodium hydrogen exchanger 3a	*D. rerio*	NM_001113473.1	Yan et al., 2007	$Na^{+,b}$ $H^{+,b}$ $Li^{+,b}$ NH_4^{+b}	
slc9a3.2	Solute carrier family 9 (sodium/hydrogen exchanger), member 3.2	Sodium hydrogen exchanger 3b	*D. rerio* *Tribolodon hakonensis*	NM_001113479.1 AB055466.1	Yan et al., 2007; Lin et al., 2008b; Liao et al., 2009; Wang et al., 2009a Hirata et al., 2003	$Na^{+,b}$ $H^{+,b}$ $Li^{+,b}$ NH_4^{+b}	
slc9a5	Solute carrier family 9 (sodium/hydrogen exchanger), member 5	Sodium hydrogen exchanger 5; sodium/hydrogen exchanger 5	*D. rerio*	NM_001113472.1	Yan et al., 2007	$Na^{+,b}$ $H^{+,b}$ $Li^{+,b}$ $NH_4^{+}(?)^b$	

slc9a6a	Solute carrier family 9 (sodium/hydrogen exchanger), member 6a	fv47b07	*D. rerio*	NM_001098256.2		Na^+,[e] K^+,[e] H^+[e]
slc9a6b	Solute carrier family 9 (sodium/hydrogen exchanger), member 6b	Sodium hydrogen exchanger 6	*D. rerio*	NM_001113476.1	Yan et al., 2007	Na^+,[e] K^+,[e] H^+[e]
slc9a7	Solute carrier family 9 (sodium/hydrogen exchanger), member 7	Solute carrier family 9, member 7	*D. rerio*	NM_001030077.2	Yan et al., 2007	Na^+,[e] K^+,[e] H^+,[e] Li^+,[e] NH_4^+(?)[e]
slc9a8	Solute carrier family 9 (sodium/hydrogen exchanger), member 8	fj61b02; sodium hydrogen exchanger 8; sodium/hydrogen exchanger 8	*D. rerio* *T. obscurus*	NM_001008586.1 AB200333.1	Yan et al., 2007; Kato et al., 2005	Na^+,[e] K^+,[e] H^+[e]

[a] From www.bioparadigms.org.
[b] Na^+/H^+ exchange (1:1).
[c] Rainbow trout.
[d] Winter flounder.
[e] $Na^+(K^+)/H^+$ exchange.

Table 10.12 The solute carrier family 10 (the sodium bile salt cotransporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc10a1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1		*Danio rerio*	XM_685653.2		Bile acids[b]
slc10a2	Solute carrier family 10 (sodium/bile acid cotransporter family), member 2	Ileal sodium/bile acid cotransporter	*D. rerio*	NM_200358.1		Bile acids[b]
slc10a4	Solute carrier family 10 (sodium/bile acid cotransporter family), member 4	Sodium/bile acid cotransporter 4	*D. rerio*	NM_001044803.1	Song et al., 2004	
zgc:92251	zgc:92251	Na(+)/bile acid cotransporter 7; sodium/bile acid cotransporter 7; solute carrier family 10, member 7	*D. rerio*	NM_001003420.1		

[a] From www.bioparadigms.org.
[b] Na^+-coupled cotransport.

Table 10.13 The solute carrier family 11 (the proton-coupled metal ion transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
nramp-alpha	Natural resistance associated macrophage protein alpha		*Oncorhynchus mykiss*	NM_001172515.1	Dorschner and Phillips, 1999; Cooper et al., 2007	Mn^{2+}, Fe^{2+} and other divalent metal ions[b]	
nramp-beta	Natural resistance associated macrophage protein beta		*O. mykiss*	NM_001172513.1	Dorschner and Phillips, 1999; Cooper et al., 2007	Mn^{2+}, Fe^{2+} and other divalent metal ions[b]	Fe^{2+},[c] Cd^{2+c}
slc11a2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	cdy; chardonnay; natural resistance-associated macrophage protein 2; nramp	*Danio rerio* *Cyprinus carpio* *Ctenopharyngodon idella* *Pimephales promelas* *Pagrus major* *Morone saxatilis* *Siniperca chuatsi* *Psetta maxima* *Paralichthys olivaceus* *Ictalurus punctatus* *Acipenser sinensis*	NM_001040370.1 AJ133735.1 GU290344.1 AF190773.2 AY485311.1 AY008746.2 HM483602.1 EU747732.1 AY907946.1 NM_001200100.1 HM217203.1	Ransom et al., 1996; Donovan et al., 2002; North and Zon, 2003; Woods et al., 2005; Cooper et al., 2006; Phillips et al., 2006; Craig et al., 2009; Sieger et al., 2009; Saeij et al., 1999; Chen et al., 2002, 2004, 2006; Burge et al., 2004	Fe^{2+},[b] Cd^{2+},[b] Co^{2+},[b] Cu^{1+},[b] Mn^{2+},[b] Ni^{2+},[b] Pb^{2+},[b] Zn^{2+b}	Fe^{2+d}
slc11 alpha	Solute carrier family 11 protein		*Takifugu rubripes*	AJ496549.1	Sibthorpe et al., 2004		
slc11 beta	Solute carrier family 11 protein		*T. rubripes*	AJ496550.1	Sibthorpe et al., 2004		

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport.
[c] Rainbow trout.
[d] Zebrafish.

Table 10.14 The solute carrier family 12 (the electroneutral cation-coupled Cl cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
nkcc2	Na-K-Cl cotransporter		*Takifugu obscurus*	AB479212.1		Sodium,[b] potassium (or ammonium),[b] chloride[b]	
nkcc2-alpha	Na/K/2Cl cotransporter (nkcc2α/BSC1α gene), isoform SLC12a1		*Anguilla anguilla* *Oreochromis mossambicus*	AJ564602.1 AY513739.1	Cutler and Cramb, 2008; Hiroi et al., 2005	Sodium,[b] potassium (or ammonium),[b] chloride[b]	
nkcc2-beta	Na/K/2Cl cotransporter		*A. anguilla* *Anguilla japonica*	AJ564603.1 AB510741.1	Cutler and Cramb, 2008	Sodium,[b] potassium (or ammonium),[b] chloride[b]	
slc12a2	Solute carrier family 12 (potassium/chloride transporters), member 2	Little ears; lte; solute carrier family 12 (potassium/chloride transporters), member 4; solute carrier family 12 member 2; zfnkcc1	*Danio rerio* *Salmo salar* *Dicentrarchus labrax* *O. mossambicus* *Sarotherodon melanotheron* *A. anguilla*	NM_001002080.1 NM_001123683.1 AY954108.1 AY513737.1 GU066877.1 AJ486858.1 AJ486859.1	Whitfield et al., 1996; Lorin-Nebel et al., 2006; Ji et al., 2008; Reynolds et al., 2008; Abbas and Whitfield, 2009; Zhang et al., 2010; Hiroi et al., 2005; Cutler and Cramb, 2002	Sodium,[b] potassium (or ammonium),[b] chloride[b]	

nkcc1-beta	Na-K-Cl cotransporter (NKCC1α)		*O. mossambicus*	AY513738.1	Hiroi et al., 2005	Sodium,[b] potassium (or ammonium),[b] chloride[b]	
slc12a3	Solute carrier family 12 (sodium/chloride transporters), member 3	Solute carrier family 12 member 3	*D. rerio* *S. salar* *Oncorhynchus mykiss* *A. anguilla* *Pseudopleuronectes americanus* *T. obscurus*	NM_001045080.1 DQ156150.1 DQ156151.1 AJ564604.1 L11615.1 AB479211.1	Wingert et al., 2007; Ji et al., 2008; Fauny et al., 2009; Wang et al., 2009a; Diep et al., 2011; Cutler and Cramb, 2008; Gamba et al., 1993	Sodium,[c] chloride[c]	Sodium,[d] chloride[d]
LOC797331	Novel protein similar to vertebrate solute carrier family 12, (potassium-chloride transporter) member 5 (SLC12A5)		*D. rerio* *D. labrax*	XM_003199112.1 FQ310506.1		Potassium (or ammonium),[e] chloride[e]	
slc12a7b	Solute carrier family 12, member 7b		*D. rerio*	XM_690968.5	Pujic et al., 2006		
slc12a8	Solute carrier family 12 (potassium/chloride transporters), member 8	Solute carrier family 12 member 8	*D. rerio*	NM_001128277.1		Polyamines (spermidine, spermine putrescine),[f] amino acids[f]	

(Continued)

Table 10.14 *(Continued)*

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc12a9	Solute carrier family 12 (potassium/chloride transporters), member 9	Solute carrier family 12 member 9	*D. rerio*	NM_001128548.1			
slc12a10.1	Solute carrier family 12 (sodium/potassium/chloride transporters), member 10.1	Solute carrier family 12, member 10.1	*D. rerio* *A. anguilla* *Anguilla japonica*	NM_001161378.1 AJ564606.1 AB510742.1	Wang et al., 2009a; Cutler and Cramb, 2008		
slc12a10.2	Solute carrier family 12 (sodium/potassium/chloride transporters), member 10.2	Solute carrier family 12 member 10.2; zNCC-like 2; znccg	*D. rerio*	NM_001045001.1	Chou et al., 2008; Hwang et al., 2009; Liao et al., 2009; Tseng et al., 2009b; Wang et al., 2009a; Janicke et al., 2010; Lee et al., 2011		
slc12a10.3	slc12a10.3 solute carrier family 12 (sodium/potassium/chloride transporters), member 10.3		*D. rerio* *O. mossambicus*	NM_001135131.1 EU518934.1	Wang et al., 2009a		

[a] From www.bioparadigms.org.
[b] Na^+/K^+(or NH_4^+)/$2Cl^-$ cotransport.
[c] Na^+/Cl^- cotransport.
[d] Winter flounder.
[e] K^+(or NH_4^+)/Cl^- cotransport.
[f] Polyamines/amino acids cotransport.

Table 10.15 The solute carrier family 13 (the Na^+–sulfate/carboxylate cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc13a1	Solute carrier family 13 (sodium/sulphate symporters), member 1	Solute carrier family 13, member 2; NaS1; NaSi-1	*Danio rerio* *Anguilla japonica*	NM_199281.1 AB111926.1	Song et al., 2004; Nakada et al., 2005; Liu et al., 2007b; Wingert et al., 2007; Markovich et al., 2008	Sulfate,[b] selenate,[b] thiosulfate[b]	Sulfate[c,d]
slc13a2	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	Solute carrier family 13, member 2	*D. rerio*	NM_213452.2		Succinate,[b] citrate,[b] α-ketoglutarate[b]	
slc13a3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	Solute carrier family 13, member 3	*D. rerio* *Pseudopleuronectes americanus* *Salmo salar*	NM_212902.1 AF102261.1 BT058913.1 BT075457.1 BT058859.1	Steffgen et al., 1999; Burckhardt et al., 2000; Burckhardt et al., 2002; Hentschel et al., 2003; Hagos et al., 2004, 2006	Succinate,[b] citrate,[b] α-ketoglutarate,[b] glutaric acid and its derivatives[b]	Succinate,[e] citrate,[e] *cis*-aconitate,[e] 2,2-dimethylsuccinate,[e] 2,3-dimethylsuccinate,[e] *meso*-2,3-dimercaptosuccinate,[e] dimercaptopropane-1-sulfonate[e]
zgc:162495	zgc:162495	SLC13A4 protein; solute carrier family 13, member 4	*D. rerio*	NM_001089551.1		Sulfate,[b] selenium and chromium oxyanions[b]	
si:ch211-221p4.4	si:ch211-221p4.4	Solute carrier family 13 (sodium-dependent citrate transporter), member 5	*D. rerio*	NM_001142566.1		Citrate,[b] succinate,[b] pyruvate[b]	

[a] From www.bioparadigms.org.
[b] Na^+-coupled cotransport.
[c] Zebrafish.
[d] Japanese eel.
[e] Winter flounder.

Table 10.16 The solute carrier family 14 (the urea transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc14a2	Solute carrier family 14 (urea transporter), member 2	Urea transporter 2	*Danio rerio* *Takifugu rubripes* *Alcolapia grahami* *Opsanus beta* *Anguilla japonica*	NM_001020519.1 NM_001032724.1 AF278537.1 AF165893.2 AB049726.1	Braun et al., 2009a, 2009b; Hong et al., 2010; Mistry et al., 2005; Walsh et al., 2000, 2001; Mistry et al., 2005	Urea[b]	Urea[c,d,e]
ut-c	Urea transporter UT-C		*A. japonica* *T. rubripes* *D. rerio*	AB181944.1 NM_001037990.1 XM_001921024.1	Walsh et al., 2001; Mistry et al., 2005	Urea[b]	Urea[e]
ut1	Urea transporter, erythrocyte		*Salmo salar*	NM_001165271.1			

[a] From www.bioparadigms.org.
[b] Facilitated transport.
[c] Lake Magadi tilapia.
[d] Gulf toadfish.
[e] Japanese eel.

Table 10.17 The solute carrier family 15 (the proton oligopeptide cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc15a1b	Solute carrier family 15 (oligopeptide transporter), member 1b	Solute carrier family 15 (oligopeptide transporter), member 1	*Danio rerio* *Salmo salar* *Chionodraco hamatus* *Gadus morhua* *Dicentrarchus labrax* *Sebastes nebulosus* *Perca flavescens* *Carassius carassius*	NM_198064.1 NM_001146682.1 AY170828.2 AY921634.1 FJ237043.2 EU160494.1 GQ906471.2 HM453869.1	Verri et al., 2003; Romano et al., 2006; Zecchin et al., 2007; Flores et al., 2008; Chen et al., 2009 Rønnestad et al., 2010; Maffia et al., 2003; Rønnestad et al., 2010; Sangaletti et al., 2009; Terova et al., 2009a	Di- and tripeptides,[b] beta-lactam antibiotics[b]	Di- and tripeptides[c,d,e,f]
slc15a2	Solute carrier family 15 (H^+/peptide transporter), member 2	PEPT2 (peptide transporter 2) solute carrier family 15 member 2	*D. rerio*	NM_001039828.1	Romano et al., 2006	Di- and tripeptides[b]	Di- and tripeptides[c]
zgc:63767	zgc:63767	bZ1L10.1 (novel peptide transporter); solute carrier family 15 member 4	*D. rerio*	NM_200957.1		Histidine,[b] di- and tripeptides[b]	

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport.
[c] Zebrafish.
[d] Atlantic salmon.
[e] Icefish.
[f] Sea bass.

Table 10.18 The solute carrier family 16 (the monocarboxylate transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc16a1	Solute carrier family 16 (monocarboxylic acid transporters), member 1		*Danio rerio* *Takifugu rubripes* *Fundulus heteroclitus*	NM_200085.1 AB574422.1 EU780698.1	Ngan and Wang, 2009	Lactate,[b] pyruvate,[b] ketone bodies[b]
slc16a3	Solute carrier family 16 (monocarboxylic acid transporters), member 3	Monocarboxylate transporter 4	*D. rerio* *T. rubripes*	NM_212708.1 AB574421.1	McDermott et al., 2007; Ngan and Wang, 2009	Lactate,[c] pyruvate,[c] ketone bodies[c]
slc16a4	Solute carrier family 16 (monocarboxylic acid transporters), member 4	Solute carrier family 16, member 4	*D. rerio*	NM_001080599.2		
slc16a8	Solute carrier family 16 (monocarboxylic acid transporters), member 8		*D. rerio*	NM_212632.1	Padhi et al., 2004	Lactate[c]
slc16a9a	Solute carrier family 16 (monocarboxylic acid transporters), member 9a		*D. rerio* *Salmo salar*	NM_200410.1 NM_001173608.1	Lagerström et al., 2005; Yang et al., 2007; Toro et al., 2009	

slc16a9b	Solute carrier family 16 (monocarboxylic acid transporters), member 9b	Solute carrier family 16 (monocarboxylic acid transporters), member 9	*D. rerio*	NM_001003552.1	Yang et al., 2007	
slc16a10	Solute carrier family 16, member 10 (aromatic amino acid transporter)	MCT 10; monocarboxylate transporter 10; solute carrier family 16 member 10	*D. rerio*	NM_001080028.1		Aromatic amino acids,[d] T3,[d] T4[d]
slc16a12a	Solute carrier family 16 (monocarboxylic acid transporters), member 12a		*D. rerio*	XM_680882.4		
slc16a12b	Solute carrier family 16 (monocarboxylic acid transporters), member 12b	MCT 12-B; monocarboxylate transporter 12-B; solute carrier family 16 member 12-B	*D. rerio*	NM_001145814.1		

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport or monocarboxylate exchange.
[c] H^+-coupled cotransport.
[d] Facilitated transport.

Table 10.19 The solute carrier family 17 (the vesicular glutamate transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence ccession ID	Reference	Predominant substrates in vertebrates[a]
slc17a5	Solute carrier family 17 (anion/sugar transporter), member 5	Sialin	*Danio rerio Salmo salar*	NM_001076727.1 NM_001173835.1		Sialic acid[b]
slc17a6a	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6a	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 like; solute carrier family 17 member 6 B; solute carrier family 17 member 6-A; vesicular glutamate transporter 2-B; vesicular glutamate transporter 2.2	*D. rerio*	NM_001009982.1	Higashijima et al., 2004b, 2004a; Kimura et al., 2006; Smear et al., 2007; Yokogawa et al., 2007; Batista and Lewis, 2008; Batista et al., 2008; Obholzer et al., 2008; Mione et al., 2008; Appelbaum et al., 2009; Bae et al., 2009; Viktorin et al., 2009; Wang, 2009; Appelbaum et al., 2010; Ito et al., 2010; Villar-Cerviño et al., 2010	Glutamate[c]

slc17a6b	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6b	Blumenkohl; protein blumenkohl; solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6; solute carrier family 17 member 6 A; solute carrier family 17 member 6-B; vesicular glutamate transporter 2-A; vesicular glutamate transporter 2.1	*D. rerio*	NM_001128821.1 NM_001005398.1	Trowe et al., 1996; Haffter et al., 1996; Neuhauss et al., 1999; Culverwell and Karlstrom, 2002; Neuhauss, 2003; Higashijima et al., 2004a, 2004b; Kimura et al., 2006; Smear et al., 2007; Yokogawa et al., 2007; Batista and Lewis, 2008; Batista et al., 2008; Mione et al., 2008; Obholzer et al., 2008; Appelbaum et al., 2009; Bae etal., 2009; Miyasaka et al., 2009; Scholpp et al., 2009; Viktorin et al., 2009; Wang, 2009; Ito et al., 2010; Kani et al., 2010; Villar-Cerviño et al., 2010; Volkmann et al., 2010; Yang et al., 2010	Glutamate[c]
slc17a7	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	Solute carrier family 17, member 7	*D. rerio*	NM_001098755.1	Higashijima et al., 2004a; Smear et al., 2007; Yokogawa et al., 2007; Obholzer et al., 2008; Bae et al., 2009; Ito et al., 2010; Villar-Cerviño et al., 2010; Volkmann et al., 2010; Rieger et al., 2009	Glutamate[c]
slc17a8	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8	Asteroid; solute carrier family 17 member 8; vesicular glutamate transporter 3	*D. rerio*	NM_001082835.1	Obholzer et al., 2008; Tanimoto et al., 2009; Trapani et al., 2009; Villar-Cerviño et al., 2010	Glutamate[c]
slc17a9a	Solute carrier family 17, member 9a		*D. rerio*	XM_001336538.2		
slc17a9b	Solute carrier family 17, member 9b		*D. rerio*	NM_001002635.1		

[a]From www.bioparadigms.org.
[b]H^+-coupled cotransport.
[c]H^+-coupled exchange.

Table 10.20 The solute carrier family 18 (the vesicular amine transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
vat1	Vesicle amine transport protein 1 homolog (T californica)	Synaptic vesicle membrane protein VAT-1 homolog	*Danio rerio*	NM_001007051.3	Loeb-Hennard et al., 2004	5HT,[b] dopamine,[b] adrenaline,[b] noradrenaline,[b] histamine[b]
slc18a2	Solute carrier family 18 (vesicular monoamine), member 2	vmat2	*D. rerio*	XM_002665190.1	Yamamoto et al., 2010; Jacobsson et al., 2010; Wen et al., 2008	5HT,[b] dopamine,[b] adrenaline,[b] noradrenaline,[b] histamine[b]
slc18a3a	Solute carrier family 18 (vesicular acetylcholine), member 3a	Probable vesicular acetylcholine transporter-A; solute carrier family 18 member 3-A	*D. rerio*	NM_001077550.1		Acetylcholine[b]
slc18a3b	Solute carrier family 18 (vesicular acetylcholine), member 3b	VAChT; probable vesicular acetylcholine transporter; probable vesicular acetylcholine transporter-B; solute carrier family 18 member 3; solute carrier family 18 member 3-B; vesicular acetylcholine transporter; zSlc18a3	*D. rerio*	NM_201107.1	Jacobsson et al., 2010	Acetylcholine[b]

[a] From www.bioparadigms.org.
[b] H^+-coupled exchange.

Table 10.21 The solute carrier family 19 (the folate/thiamine transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Predominant substrates in vertebrates[a]
slc19a3	Solute carrier family 19, member 3	Thiamine transporter 2	*Danio rerio*	NM_001044707.1	Thiamine

[a] From www.bioparadigms.org.

Table 10.22 The solute carrier family 20 (the type III Na^+–phosphate cotransporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc20a1a	Solute carrier family 20, member 1a	Sodium-dependent phosphate transporter 1-A; solute carrier family 20 member 1-A	*Danio rerio* *Salmo salar*	NM_213179.1 NM_001140224.1	Song et al., 2004; Nichane et al., 2006; Wingert et al., 2007; Tan et al., 2010	Phosphate[b]
slc20a1b	Solute carrier family 20, member 1b	Sodium-dependent phosphate transporter 1-B; solute carrier family 20 (phosphate transporter), member 1; solute carrier family 20 member 1-B	*D. rerio*	NM_212588.1	Woods et al., 2005; Nichane et al., 2006; Yang et al., 2007	Phosphate[b]
zgc: 152990	zgc: 152990	fi23g11; sodium-dependent phosphate transporter 2; solute carrier family 20, member 2	*D. rerio*	NM_001077546.1		Phosphate[b]

[a] From www.bioparadigms.org.
[b] Na^+-coupled cotransport.

Table 10.23 The solute carrier family 21 (the organic anion transporting family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slco1c1	Solute carrier organic anion transporter family, member 1C1	oatp1c1; solute carrier organic anion transporter family member 1C1; zOatp1c3	*Danio rerio*	NM_001044997.3	Meier-Abt et al., 2005; Popovic et al., 2010	T4, T3, rT3, BSP
LOC100536648	Solute carrier organic anion transporter family member 1C1-like		*D. rerio*	XM_003198547.1		
slco1d1	Solute carrier organic anion transporter family, member 1D1	Oatp1d1	*D. rerio*	NM_001089333.1	Popovic et al., 2010	
slco1f1	Solute carrier organic anion transporter family, member 1F1	Oatp1f1	*D. rerio*	NM_212917.1	Popovic et al., 2010	
slco1f2	Solute carrier organic anion transporter family, member 1F2	Oatp1f2	*D. rerio*	NM_001128273.1	Popovic et al., 2010	
slco1f3	Solute carrier organic anion transporter family, member 1F3	Oatp1f3	*D. rerio*	NM_001135684.1	Popovic et al., 2010	
slco1f4	Solute carrier organic anion transporter family, member 1F4	Oatp1f4	*D. rerio*	NM_001080666.1	Popovic et al., 2010	
slco2a1	Solute carrier organic anion transporter family, member 2A1	Oatp2a1; zOatp2a3	*D. rerio*	NM_001089582.1	Lin et al., 2010; Popovic et al., 2010	Prostaglandin (lactate[b])
slco2b1	Solute carrier organic anion transporter family, member 2B1	Oatp2b1; solute carrier organic anion transporter family, member 2b1	*D. rerio*	NM_001037678.2	Popovic et al., 2010	E-3-S, DHEAS, BSP

slco3a1	Solute carrier organic anion transporter family, member 3A1	Oatp3a1; zOatp3a2	*D. rerio*	NM_001045188.2	Popovic et al., 2010; Meier-Abt et al., 2005	E-3-S, prostaglandin
LOC570448	Solute carrier organic anion transporter family member 3A1-like		*D. rerio*	XM_693928.2		
slco4a1	Solute carrier organic anion transporter family, member 4A1	Oatp4a1; zOatp4a2	*D. rerio*	XM_691171.2	Meier-Abt et al., 2005; Popovic et al., 2010	Taurocholate, T3, prostaglandin
slco5a1	Solute carrier organic anion transporter family, member 5A1	Oatp5a2	*D. rerio* *Dicentrarchus labrax*	XM_693463.5 CBN81921.1	García-Lecea et al., 2008; Popovic et al., 2010	
LOC556735	Solute carrier organic anion transporter family member 5A1-like		*D. rerio*	XM_679609.4		

[a]From www.bioparadigms.org.
[b]Cotransport.

Table 10.24 The solute carrier family 22 (the organic cation/anion/zwitterion transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc22a4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	Solute carrier family 22 member 4	*Danio rerio* *Salmo salar*	NM_200849.1 BT072375.1	Nilsson et al., 2009	Ergothioneine,[b] organic cations,[c] polyspecific[c]	
LOC100000685	Similar to OCTN2 protein		*D. rerio*	XM_001340800.2	Nilsson et al., 2009		
oat	Organic anion transporter	ROAT1; organic anion transporter 1; renal organic anion transporter 1; solute carrier family 22 member 6; Oat	*D. rerio* *S. salar* *Pseudopleuronectes americanus*	NM_207077.1 NM_001140144.1 Z97028.1	Aslamkhan et al., 2006 Wolff et al., 1997; Aslamkhan et al., 2006	Organic anions,[d] polyspecific,[d] (PAH, α-keto glutarate, etc.)[d]	PAH,[e] estrone sulfate,[e] adefovir,[e] 2,4-DPA[e]
oatx	Organic anion transporter X		*D. rerio*	XM_001923093.3	Aslamkhan et al., 2006		

s226a	Solute carrier family 22 member 6-A		*S. salar*	NM_001139884.1	
slc22a7a	Solute carrier family 22 (organic anion transporter), member 7a	Solute carrier family 22 member 7	*D. rerio*	NM_001083861.1	Organic anions,[c,d] polyspecific,[c,d] (cGMP)[c,d]
slc22a7b	Solute carrier family 22 (organic anion transporter), member 7b	Solute carrier family 22, member 7-like	*D. rerio* *S. salar*	NM_200349.1 BT046087.1	
slc22a18	Solute carrier family 22 (organic cation transporter), member 18	Solute carrier family 22 member 18	*D. rerio* *S. salar*	NM_001037385.1 NM_001173968.1	(Chloroquine, quinidine)

[a] From www.bioparadigms.org.
[b] Cotransport.
[c] Facilitated transport.
[d] Organic anion exchange.
[e] Winter flounder.

Table 10.25 The solute carrier family 23 (the Na^+-dependent ascorbic acid transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc23a1	Solute carrier family 23 (nucleobase transporters), member 1	Solute carrier family 23 member 1	*Danio rerio*	NM_001173499.1		L-ascorbic acid[b]
slc23a2	Solute carrier family 23 (nucleobase transporters), member 2		*D. rerio*	XM_002663198.1		L-ascorbic acid[b]

[a]From www.bioparadigms.org.
[b]Na^+-coupled cotransport.

Table 10.26 The solute carrier family 24 (the $Na^+/(Ca^{2+}\text{-}K^+)$ exchanger family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc24a1	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 1		*Danio rerio* *Morone saxatilis*	XM_001921942.1 EF076649.1		Na^+,[b] Ca^{2+},[b] K^+[b]
slc24a3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3		*D. rerio*	XM_680210.3		Na^+,[b] Ca^{2+},[b] K^+[b]
slc24a5	Solute carrier family 24, member 5	K-dependent Na,Ca exchanger 5; Na(+)/K(+)/Ca(2+)-exchange protein 5; gol; gol-1; golden; golden-1; protein golden; sodium/potassium/calcium exchanger 5	*D. rerio* *Nothobranchius furzeri*	NM_001030280.1 FJ828657.1	Lamason et al., 2005; Müller and Kelsh, 2006; Braasch et al., 2007; Brown et al., 2007; Yeyati et al., 2007; Doyon et al., 2008; Wang et al., 2009b; Wittkopp et al., 2009	Na^+,[b] Ca^{2+},[b] K^+[b]

[a] From www.bioparadigms.org.
[b] K^+-dependent, Na^+/Ca^{2+} exchange.

Table 10.27 The solute carrier family 25 (the mitochondrial carrier family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc25a1	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	Solute carrier family 25, member 1	*Danio rerio*	NM_200607.1	Sreenivasan et al., 2008	Citrate,[b] malate,[b] PEP[b]
LOC795332	Similar to tricarboxylate transport protein, mitochondrial precursor (citrate transport protein, CTP) (tricarboxylate carrier protein) (solute carrier family 25 member 1)	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	*D. rerio* *Anguilla anguilla* *Ictalurus punctatus* *I. furcatus* *Tetraodon nigroviridis*	XM_003199276.1 AJ744769.1 NM_001200486.1 GU588657.1 GU588110.1 CAAE01014764.1	Zara et al., 2007	Citrate,[b] malate,[b] PEP[b]
slc25a3a	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3a		*D. rerio*	NM_200715.1 BC059476.1 BX649476.3		Phosphate[c]
slc25a3b	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3b	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 3; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; solute carrier family 25 member 3	*D. rerio* *Salmo salar* *Osmerus mordax*	NM_213722.1 BC067565.1 BC046007.1 BT060336.1 BT058623.1 BT074720.1 BT075300.1		Phosphate[c]
slc25a4	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	ADP/ATP translocase 1	*D. rerio*	NM_214702.1	Kolmakov et al., 2008; Johnston et al., 2009	ADP,[d] ATP[d]

slc25a5	Solute carrier family 25 alpha, member 5	ADP/ATP translocase 2	*D. rerio* *Oncorhynchus mykiss* *S. salar* *O. mordax* *Esox lucius*	NM_173247.1 FJ591153.1 BT060312.1 BT060089.1 BT075713.1 BT074758.1 BT079926.1	Padhi et al., 2004; Vihtelic et al., 2005; Whitehead et al., 2005	ADP,[d] ATP[d]
slc25a6	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	Adenine nucleotide translocator 3	*D. rerio*	NM_001128732.1	Liongue and Ward, 2007	ADP,[d] ATP[d]
ucp1	Uncoupling protein 1	Mitochondrial uncoupling protein 3; uncoupling protein 4	*D. rerio* *Cyprinus carpio* *Sparus aurata* *Siniperca chuatsi*	NM_199523.2 AY461434.2 FJ710211.1 EU719621.1	Stuart et al., 2001; Jastroch et al., 2005; Harden et al., 2006; Jastroch et al., 2008; Klingenspor et al., 2008; Saito et al., 2008; Lam et al., 2008 Jastroch et al., 2005 Bermejo-Nogales et al., 2010	H^{+e}
ucp2	Uncoupling protein 2	UCP 2; mitochondrial uncoupling protein 2; solute carrier family 25 member 8; UCP2	*D. rerio* *C. carpio* *S. salar* *Leuciscus cephalus* *Epinephelus coioides* *S. chuatsi* *O. mordax* *Zoarces viviparus* *Pachycara brachycephalum* *Hypophthalmichthys molitrix* *Ctenopharyngodon idella*	NM_131176.1 AJ243486.1 BT047080.1 AY368268.1 HM037342.1 EU719621.1 EU719622.1 BT075349.1 AY625191.1 AY625190.1 EF100900.1 AY948546.1	Stuart et al., 1999; Stuart et al., 2001; Jastroch et al., 2005; Jastroch et al., 2008; Liu et al., 2008; Saito et al., 2008; Shieh et al., 2010 Stuart et al., 1999 Mark et al., 2006 Mark et al., 2006 Liao et al., 2006 Liao et al., 2006	H^{+e}
ucp2a	Uncoupling protein 2A		*O. mykiss*	NM_001124654.1	Coulibaly et al., 2006	H^{+e}

(Continued)

Table 10.27 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
ucp2b	Uncoupling protein 2B		*O. mykiss*	NM_001124571.1	Coulibaly et al., 2006	H^{+e}
ucp3	Uncoupling protein 3	Uncoupling protein 2, like	*D. rerio* *S. aurata*	NM_200353.1 EU555336.1	Jastroch et al., 2005; Saito et al., 2008 Bermejo-Nogales et al., 2010	H^{+e}
slc25a10	Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	Mitochondrial dicarboxylate carrier	*D. rerio*	NM_201172.1		Malate,[f] phosphate,[f] succinate,[f] sulphate,[f] thiosulphate[f]
slc25a11	Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	Mitochondrial 2-oxoglutarate/malate carrier protein	*D. rerio* *I. punctatus*	NM_001002099.1 NM_001201090.1	Hoffmann et al., 2008	2-Oxoglutarate,[g] malate[g]
slc25a12	Solute carrier family 25 (mitochondrial carrier, Aralar), member 12	Calcium-binding mitochondrial carrier protein Aralar1	*D. rerio* *S. salar*	NM_212782.1 BT057771.1 BT045815.1		Aspartate,[h] glutamate[h]
aralar2			*E. lucius*	BT079505.1		Aspartate,[h] glutamate[h]
slc25a14	Solute carrier family 25 (mitochondrial carrier, brain), member 14	Brain mitochondrial carrier protein 1	*D. rerio*	NM_200164.1	Song et al., 2004	
ornt1	Mitochondrial ornithine transporter 1		*I. punctatus* *S. salar* *Anoplopoma fimbria*	NM_001200508.1 BT057012.1 BT083167.1		Ornithine,[i] citrulline,[i] lysine,[i] arginine[i]
slc25a15a	Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15°		*D. rerio*	NM_001080638.1		Ornithine,[i] citrulline,[i] lysine,[i] arginine[i]

slc25a16	Solute carrier family 25, member 16		*D. rerio*	NM_205549.1		
zgc:111878	zgc:111878	Mitochondrial thiamine pyrophosphate carrier; solute carrier family 25 member 19	*D. rerio*	NM_205715.1	Song et al., 2004	Thiamine pyrophosphate,[j] thiamine monophosphate,[j] (deoxy) nucleotides[j]
slc25a20	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	Mitochondrial carnitine/acylcarnitine carrier protein	*D. rerio* *S. salar* *O. mordax*	NM_200859.1 BT044930.1 BT075584.1		Carnitine,[k] acylcarnitine[k]
slc25a21	Solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21	Mitochondrial 2-oxodicarboxylate carrier	*D. rerio*	NM_001076632.1	Sreenivasan et al., 2008	oxoadipate,[l] oxoglutarate[l]
slc25a22	Solute carrier family 25 (mitochondrial carrier: glutamate), member 22	cb229	*D. rerio* *I. punctatus* *I. furcatus*	NM_213408.1 NM_001200865.1 GU588225.1	Song et al., 2004; Lv et al., 2008	Glutamate[m]
zgc:92470	zgc:92470	Calcium-binding mitochondrial carrier protein SCaMC-1; small calcium-binding mitochondrial carrier protein 1; solute carrier family 25 member 24	*D. rerio* *S. salar*	NM_001004606.1 BT045397.1	Song et al., 2004	ATP-Mg^{2+},[n] ATP,[n] ADP,[n] AMP,[n] Pi[n]

(Continued)

Table 10.27 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc25a25a	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25a	Calcium-binding mitochondrial carrier protein SCaMC-2-A; small calcium-binding mitochondrial carrier protein 2-A; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25; solute carrier family 25 member 25; solute carrier family 25 member 25-A	*D. rerio*	NM_213257.1	Song et al., 2004; Woods et al., 2005; Petko et al., 2009	
slc25a25b	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25b	Calcium-binding mitochondrial carrier protein SCaMC-2-B; small calcium-binding mitochondrial carrier 2; small calcium-binding mitochondrial carrier protein 2-B; solute carrier family 25 member 25-B; solute carrier family 25, member 25	*D. rerio*	NM_001160020.1		
slc25a26	Solute carrier family 25, member 26	*S*-adenosylmethionine mitochondrial carrier protein; mitochondrial *S*-adenosylmethionine transporter	*D. rerio* *S. salar* *I. punctatus*	NM_001030143.1 BT046569.1 NM_001200801.1		*S*-adenosyl-methionine,[o] *S*-adenosyl-homocysteine[o]
slc25a27	Solute carrier family 25, member 27	Mitochondrial uncoupling protein 4	*D. rerio* *I. furcatus*	NM_200341.1 HP430492.1		
slc25a28	Solute carrier family 25, member 28	mfrn2; mitochondrial iron transporter 2; mitoferrin 2; mitoferrin-2	*D. rerio* *S. salar*	NM_213119.1 NM_001173593.1	Woods et al., 2005; Shaw et al., 2006; Metzendorf et al., 2009	Fe^{2+}

slc25a29	Solute carrier family 25, member 29	Mitochondrial carnitine/acylcarnitine carrier protein CACL	*D. rerio*	NM_001030237.1		Ornithine, acylcarnitine
slc25a32a	Solute carrier family 25, member 32a	Mitochondrial folate transporter/carrier	*D. rerio*	NM_200256.1	Song et al., 2004	Folate
slc25a32b	Solute carrier family 25, member 32b	Mitochondrial folate transporter/carrier	*D. rerio*	NM_001013336.1		Folate
slc25a33	Solute carrier family 25, member 33	Mitochondrial carrier protein; solute carrier family 25 member 33	*D. rerio*	NM_213157.1	Woods et al., 2005	UTP
zgc:65857	zgc:65857	Solute carrier family 25 member 34	*D. rerio*	NM_200580.1		
si:ch211-268m12.5	si:ch211-268m12.5	Solute carrier family 25 member 35	*D. rerio*	NM_001099447.1		
slc25a36a	Solute carrier family 25, member 36a	DKEYP-90G12.1; solute carrier family 25 member 36-A	*D. rerio*	NM_001002667.1		
slc25a36b	Solute carrier family 25, member 36b		*D. rerio*	CK688816.1		
slc25a37	Solute carrier family 25, member 37	Erythroid-specific mitochondrial mitoferrin; frascati; frs; mfrn; mfrn1; mitochondrial iron transporter 1; mitoferrin; mitoferrin-1; protein frascati	*D. rerio*	NM_001039971.1	Ransom et al., 1996; Shaw et al., 2006; Metzendorf et al., 2009; Amigo et al., 2009	Fe^{2+}
slc25a38a	Solute carrier family 25, member 38a	Solute carrier family 25 member 38-A	*D. rerio*	NM_001076602.1	Guernsey et al., 2009	Glycine (?)
slc25a38b	Solute carrier family 25, member 38a	Solute carrier family 25 member 38-B	*D. rerio*	NM_001202458.1	Guernsey et al., 2009	Glycine (?)
slc25a39	Solute carrier family 25, member 39	Solute carrier family 25 member 39	*D. rerio*	NM_200486.1	Song et al., 2004; Nilsson et al., 2009	

(Continued)

Table 10.27 (*Continued*)

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc25a40	Solute carrier family 25, member 40	Solute carrier family 25 member 40	*D. rerio*	NM_001002360.1		
slc25a42	Solute carrier family 25, member 42	Solute carrier family 25 member 42	*D. rerio*	NM_001045453.1		
slc25a43	Solute carrier family 25, member 43	Mitochondrial carrier; solute carrier family 25 member 43	*D. rerio*	NM_001004497.2	Woods et al., 2005	
slc25a44a	Solute carrier family 25, member 44 a		*D. rerio*	NM_001007350.1		
slc25a44b	Solute carrier family 25, member 44 b		*D. rerio*	NM_001013519.1		
slc25a46	Solute carrier family 25, member 46	Solute carrier family 25 member 46	*D. rerio*	NM_001002558.1		
slc25a47a	Solute carrier family 25, member 47a	Hepatocellular carcinoma downregulated mitochondrial carrier homolog A; solute carrier family 25 member 47-A; solute carrier family 25, member 47	*D. rerio*	NM_001045314.1		

slc25a47b	Solute carrier family 25, member 47b	Hepatocellular carcinoma downregulated mitochondrial carrier homolog B; solute carrier family 25 member 47-B	*D. rerio*	NM_001089581.1
zgc:92090	zgc:92090	Mitochondrial carrier protein-like; putative mitochondrial carrier protein FLJ44862 homolog; solute carrier family 25 member 48	*D. rerio*	NM_001002367.1

[a] From www.bioparadigms.org.
[b] Citrate-H^+/malate exchange.
[c] H^+-coupled cotransport; phosphate/OH^- exchange.
[d] ATP/ADP exchange.
[e] Facilitated transport.
[f] Malate/phosphate exchange.
[g] 2-Oxoglutarate/malate exchange.
[h] Aspartate/glutamate-H^+ exchange.
[i] Ornithine/citrulline-H^+ exchange; ornithine/H^+ exchange.
[j] Thiamine pyrophosphate/thiamine monophosphate exchange; thiamine pyrophosphate/(deoxy)nucleotide exchange.
[k] Carnitine/acylcarnitines exchange; facilitated transport (at slow rate).
[l] Oxoadipate/oxoglutarate exchange.
[m] H^+-dependent cotransport; glutamate/OH^- exchange.
[n] ATP-Mg^{2+}/Pi exchange.
[o] *S*-adenosyl-methionine/*S*-adenosyl-homocysteine exchange.

Table 10.28 The solute carrier family 26 (the multifunctional anion exchanger family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc26a1	Solute carrier family 26 (sulfate transporter), member 1	Sat1; sulfate anion transporter 1	*Danio rerio* *Oncorhynchus mykiss* *Anguilla japonica*	NM_001080667.1 NM_001124486.1 AB111927.1	Van Campenhout et al., 2006; Gorelick et al., 2008; Bayaa et al., 2009; Hong et al., 2010 Katoh et al., 2006 Nakada et al., 2005	SO_4^{2-},[b] HCO_3^-,[b] oxalate[b]	SO_4^{2-},[c] Cl^-[c]
slc26a3	Solute carrier family 26, member 3		*D. rerio* *A. japonica* *Takifugu obscurus*	NM_001135683.1 AB111930.1 AB200326.1	Bayaa et al., 2009; Perry et al., 2009 Kato et al., 2005	HCO_3^-,[b] Cl^-,[b] SO_4^{2-},[b] oxalate	
slc26a4	Solute carrier family 26, member 4	Anion exchanger SLC26A4; pendrin	*D. rerio*	NM_001165915.1	Bayaa et al., 2009; Perry et al., 2009	I^-,[b] HCO_3^-,[b] Cl^-,[b] formate[b]	
slc26a5	Solute carrier family 26, member 5	Prestin	*D. rerio* *T. obscurus*	NM_201473.1 AB200327.1	Song et al., 2004; Weber et al., 2003; Tang et al., 2005; Albert et al., 2007; Schaechinger and Oliver, 2007; Bayaa et al., 2009; Tan et al., 2011 Kato et al., 2005		

slc26a6	Solute carrier family 26, member 6		*A. japonica*	AB084425.1		Cl^{-},[b] HCO_3^{-},[b] oxalate,[b] SO_4^{2-},[b] formate[b]	
slc26a6a	Solute carrier family 26, member 6 a		*Opsanus beta* *T. obscurus*	EF529734.1 AB200328.1	Grosell et al., 2009; Kato et al., 2005	Cl^{-},[b] HCO_3^{-},[b] oxalate,[b] SO_4^{2-},[b] formate[b]	HCO_3^{-}[d]
slc26a6b	Solute carrier family 26, member 6 b		*A. japonica* *T. obscurus*	AB111928.1 AB200329.1	Kato et al., 2005	Cl^{-},[b] HCO_3^{-}, oxalate,[b] SO_4^{2-},[b] formate[b]	HCO_3^{-}[d]
slc26a6l	Solute carrier family 26, member 6, like	slc26a6c; solute carrier family 26, member 6	*D. rerio* *A. japonica* *T. obscurus*	NM_001114417.1 AB111929.1 AB200330.1	Bayaa et al., 2009; Perry et al., 2009; Kato et al., 2005		
slc26a11	Solute carrier family 26, member 11	Sodium-independent sulfate anion transporter	*D. rerio* *T. obscurus*	NM_199767.1 AB200331.1	Song et al., 2004; Bayaa et al., 2009 Kato et al., 2005	SO_4^{2-}[b]	

[a] From www.bioparadigms.org.
[b] Anion exchange.
[c] Japanese eel.
[d] Mefugu.

Table 10.29 The solute carrier family 27 (the fatty acid transporter family). No substrates has been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc27a1a	Solute carrier family 27 (fatty acid transporter), member 1a	Solute carrier family 27 (fatty acid transporter), member 1	*Danio rerio*	NM_001013537.1		Long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA)[b]
slc27a1b	Solute carrier family 27 (fatty acid transporter), member 1b	Solute carrier family 27 (fatty acid transporter), member 1	*D. rerio*	NM_001077248.1		long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA)[b]
slc27a2	Solute carrier family 27 (fatty acid transporter), member 2	fb99g05	*D. rerio* *Salmo salar*	NM_001025299.1 BT059131.1	Lam et al., 2006	Long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA)[b]
slc27a4	Solute carrier family 27 (fatty acid transporter), member 4		*D. rerio*	NM_001017737.1		Long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA)[b]
slc27a6	Solute carrier family 27 (fatty acid transporter), member 6		*D. rerio*	NM_001077386.1		Long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA)[b]

[a]From www.bioparadigms.org.
[b]VLCFA-activated LCFA transport.

Table 10.30 The solute carrier family 29 (the facilitative nucleoside transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc29a4	Solute carrier family 29 (nucleoside transporters), member 4	Equilibrative nucleoside transporter 4; solute carrier family 29 member 4	*Danio rerio*	NM_001080572.1	Sivasubbu et al., 2006	Adenosine[b]

[a]From www.bioparadigms.org.
[b]Unknown.

Table 10.31 The solute carrier family 30 (the zinc efflux family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc30a1	Solute carrier family 30 (zinc transporter), member 1	ZnT1; zinc transporter 1	*Danio rerio* *Takifugu rubripes* *Oncorhynchus mykiss* *Cyprinus carpio*	NM_200879.1 NM_001032723.1 NM_001124528.1 AY691190.1	Feeney et al., 2005; Kambe et al., 2006; Zheng et al., 2008; Zheng et al., 2010a; Balesaria and Hogstrand, 2006; Muylle et al., 2006	Zinc[b]	Zinc[c]
slc30a2	Solute carrier family 30 (zinc transporter), member 2	ZnT2; novel zinc transporter protein; zinc transporter 2	*D. rerio*	NM_001045020.1	Feeney et al., 2005; Kambe et al., 2006; Zheng et al., 2008	Zinc[b]	–
slc30a4	Solute carrier family 30 (zinc transporter), member 4	ZnT4; zinc transporter 4	*D. rerio* *Salmo salar*	NM_200643.1 NM_001139905.1	Feeney et al., 2005; Kambe et al., 2006	Zinc[b]	–
slc30a5	Solute carrier family 30 (zinc transporter), member 5	ZnT5; solute carrier family 30 member 5; zinc transporter 5; znT-5	*D. rerio*	NM_001002322.1	Feeney et al., 2005; Kambe et al., 2006; Zheng et al., 2008	Zinc[b]	–
slc30a6	Solute carrier family 30 (zinc transporter), member 6	ZnT6; solute carrier family 30 member 6; zinc transporter 6; znT-6	*D. rerio* *S. salar*	NM_205651.1 NM_001139824.1	Feeney et al., 2005; Kambe et al., 2006	Zinc[b]	–
slc30a7	Solute carrier family 30 (zinc transporter), member 7	Solute carrier family 30 member 7; zinc transporter 7	*D. rerio* *S. salar*	NM_001100086.1 BT045928.1	Feeney et al., 2005; Kambe et al., 2006	Zinc[b]	–
slc30a9	Solute carrier family 30 (zinc transporter), member 9	Solute carrier family 30 member 9; zinc transporter 9; znT-9	*D. rerio*	NM_001008575.1	Feeney et al., 2005; Kambe et al., 2006	Unknown	–
slc30a10	Solute carrier family 30 (zinc transporter), member 10	ZnT10; zinc transporter 10	*D. rerio*	NM_001128234.1	Kambe et al., 2006	–	–

[a] From www.bioparadigms.org.
[b] Unknown.
[c] Fugu.

Table 10.32 The solute carrier family 31 (the copper transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc31a1	Solute carrier family 31 (copper transporters), member 1	ctr-1; ctr1; high affinity copper uptake protein	*Danio rerio* *Sparus aurata* *Platichthys flesus* *Salmo salar*	NM_205717.2 AJ630205.3 AJ632131.1 NM_001173944.1	Mackenzie et al., 2004; Rawls et al., 2004; Craig et al., 2007; Griffitt et al., 2007; Lam et al., 2008; Craig et al., 2009; Minghetti et al., 2008	Cu^+,[b] cisplatin[b]
copt2	Probable low affinity copper uptake protein 2	Solute carrier family 31 member 2	*S. salar*	NM_001165291.1		Copper,[c] cisplatin,[c] carboplatin[c]

[a] From www.bioparadigms.org.
[b] Energy-independent, K^+-dependent transport.
[c] Unknown.

Table 10.33 The solute carrier family 32 (the vesicular inhibitory amino acid transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc32a1	Solute carrier family 32 (GABA vesicular transporter), member 1	viaat	*Danio rerio* *Salmo salar*	NM_001080701.1 NM_001165390.1	Kimura et al., 2006	GABA,[b] glycine[b]

[a]From www.bioparadigms.org.
[b]H^+-coupled exchange.

Table 10.34 The solute carrier family 33 (the acetyl-CoA transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc33a1	Solute carrier family 33 (acetyl-CoA transporter), member 1		*Danio rerio*	NM_201108.1	Lin et al., 2008a	Acetyl-CoA[b]

[a]From www.bioparadigms.org.
[b]Facilitated transport.

Table 10.35 The solute carrier family 34 (the type II Na^+–phosphate cotransporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
npt2a	Sodium-dependent phosphate transport protein 2A		*Salmo salar*	NM_001140086.1		Inorganic phosphate[b]	
slc34a2a	Solute carrier family 34 (sodium phosphate), member 2a	Na/Pi cotransport system protein, type II	*Danio rerio*	NM_131624.1	Nalbant et al., 1999; Werner et al., 2002; Graham et al., 2003; Carlile et al., 2008	Inorganic phosphate[b]	Inorganic phosphate[c]
slc34a2b	Solute carrier family 34 (sodium phosphate), member 2b	Sodium-dependent phosphate transport protein 2B	*D. rerio* *Cyprinus carpio* *Pseudopleuronectes americanus*	NM_182877.1 AF297189.1 U13963.1	Graham et al., 2003; Woods et al., 2005; Carlile et al., 2008; Werner and Kinne, 2001; Werner et al., 1994; Kohl et al., 1996; Forster et al., 1997; Elger et al., 1998; Huelseweh et al., 1998; Kohl et al., 1998; Forster et al., 2000; Werner and Kinne, 2001; Werner et al., 2002; Graham et al., 2003; Forster et al., 2006; Virkki et al., 2006; Bacconi et al., 2007; McHaffie et al., 2007	Inorganic phosphate[b]	Inorganic phosphate[c,d]

[a] From www.bioparadigms.org.
[b] Na^+-dependent cotransport.
[c] Zebrafish.
[d] Winter flounder.

Table 10.36 The solute carrier family 35 (the nucleotide-sugar transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc35a1	CMP-sialic acid transporter		*Salmo salar* *Takifugu rubripes* *Oryzias latipes* *Osmerus mordax*	NM_001139611.1 AJ705103.1 NM_001104842.1 BT075035.1		CMP-sialic acid[b]
slc35a2	Solute carrier family 35 (UDP-galactose transporter), member 2	UDP-galactose transporter	*Danio rerio*	AL590148.8		UDP-galactose,[c] UDP-N-acetylgalactosamine[c]
s35a3	UDP-N-acetylglucosamine transporter		*S. salar* *D. rerio*	NM_001140128.1 NM_001123249.1		UDP-N-acetylglucosamine[c]
zgc:153507	zgc:153507	Probable UDP-sugar transporter protein SLC35A4; solute carrier family 35 member A4	*D. rerio*	NM_001077736.1		
slc35a5	Solute carrier family 35, member A5	Probable UDP-sugar transporter protein SLC35A5	*D. rerio* *S. salar*	NM_200654.1 NM_001173672.1		
slc35b1	Solute carrier family 35, member B1	Solute carrier family 35 member B1	*D. rerio* *S. salar*	NM_001004583.1 NM_001140406.1		
slc35b2	Solute carrier family 35, member B2	Adenosine 3′-phospho 5′-phosphosulfate transporter 1; papst1; pic; pinscher	*D. rerio*	NM_205635.1	Karlstrom et al., 1996; Culverwell and Karlstrom, 2002; Neuhauss, 2003; Clément et al., 2008	PAPS
slc35b3	Solute carrier family 35, member B3	PAPST2; adenosine 3′-phospho 5′-phosphosulfate transporter 2	*D. rerio*	NM_001039995.1	Clément et al., 2008	PAPS

slc35b4	Solute carrier family 35, member B4	UDP-xylose and UDP-*N*-acetylglucosamine transporter	*D. rerio* *S. salar*	NM_212652.1 NM_001142712.1		UDP-xylose, UDP-*N*-acetylglucosamine
slc35c1	Solute carrier family 35, member C1	GDP-fucose transporter 1; fc03b12	*D. rerio*	NM_001008590.1	Chou et al., 2008	GDP-fucose[d]
slc35c2	Solute carrier family 35, member C2	Ovarian cancer overexpressed 1; solute carrier family 35 member C2	*D. rerio*	NM_212643.1		
slc35d1a	Solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1a	UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter; solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1	*D. rerio*	NM_001003877.1	Amsterdam et al., 2004; Liongue and Ward, 2007	UDP-glucuronic acid,[e] UDP-*N*-acetylgalactosamine[e]
slc35d1b	Solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1b		*D. rerio* *O. mordax*	NM_200475.1 BT075638.1		UDP-glucuronic acid,[e] UDP-*N*-acetylgalactosamine[e]
s35d2		UDP-*N*-acetylglucosamine/UDP-glucose/GDP-mannose transporter	*Esox lucius* *Anoplopoma fimbria*	BT079765.1 BT082497.1		

(Continued)

Table 10.36 *(Continued)*

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc35e1	Solute carrier family 35, member E1	Solute carrier family 35 member E1	*D. rerio*	NM_213074.1		
s35e2		Solute carrier family 35 member E2	*S. salar*	NM_001140243.1		
slc35e2b	Solute carrier family 35, member E2B		*D. rerio*	XM_001336210.2		
slc35e3	Solute carrier family 35, member E3	Solute carrier family 35 member E3	*D. rerio*	NM_001030179.1		
slc35f1	Solute carrier family 35, member F1		*D. rerio*	XM_677843.3		
slc35f2	Solute carrier family 35, member F2		*D. rerio*	NM_001076556.1		
slc35f3	Solute carrier family 35, member F3		*D. rerio*	NM_001111205.1		
s35f4	Solute carrier family 35 member F4		*S. salar*	NM_001173862.1		

[a] From www.bioparadigms.org.
[b] CMP-coupled exchange.
[c] UMP-coupled exchange.
[d] GMP-coupled exchange.
[e] Exchange.

Table 10.37 The solute carrier family 36 (the proton-coupled amino acid transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc36a1	Solute carrier family 36 (proton/amino acid symporter), member 1	–	*Danio rerio*	XM_682640.4	–	Gly,[b] Ala,[b] Pro,[b] GABA,[b] D-Ala,[b] MeAIB,[b] taurine[b]

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport.

Table 10.38 The solute carrier family 37 (the sugar–phosphate/phosphate exchanger family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc37a2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	Solute carrier family 37 member 2; sugar–phosphate exchanger 2	*Danio rerio*	NM_213014.1	Woods et al., 2005; Hoffmann et al., 2008	Glycerol 3-phosphate[b]
slc37a4a	Solute carrier family 37 (glucose-6-phosphate transporter), member 4a	fc25d06; glucose-6-phosphate translocase; glycerol-6-phosphate transporter; solute carrier family 37 (glucose-6-phosphate transporter), member 4	*D. rerio*	NM_214738.1		Glucose-6-phosphate[c]
slc37a4b	Solute carrier family 37 (glucose-6-phosphate transporter), member 4b		*D. rerio*	NM_200940.1		Glucose-6-phosphate[c]

[a] From www.bioparadigms.org.
[b] Unknown.
[c] Inorganic phosphate-coupled exchange.

Table 10.39 The solute carrier family 38 (the family of system A and system N sodium-coupled neutral amino acid transporters). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
System A subfamily						
si:ch211-132p20.4	si:ch211-132p20.4	Amino acid transporter A2; sodium-coupled neutral amino acid transporter 2; solute carrier family 38 member 2; solute carrier family 38, member 2 like; system A amino acid transporter 2; system A transporter 1; system N amino acid transporter 2	*Danio rerio*	NM_001045104.1		Ala,[b] Asn,[b] Cys,[b] Gln,[b] Gly,[b] His,[b] Met,[b] Pro,[b] Ser[b]
slc38a4	Solute carrier family 38, member 4		*D. rerio*	NM_001005944.1	Cheng et al., 2006; Jima et al., 2009	Ala,[b] Asn,[b] Cys,[b] Gly,[b] Ser,[b] Thr[b]
System N subfamily						
slc38a3	Solute carrier family 38, member 3		*D. rerio*	NM_001002648.1	Johnston et al., 20093	Gln,[c] His,[c] Ala,[c] Asn[c]

(Continued)

Table 10.39 *(Continued)*

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
Orphan members						
slc38a6	Solute carrier family 38, member 6	Na(+)-coupled neutral amino acid transporter 6; probable sodium-coupled neutral amino acid transporter 6	*D. rerio*	NM_001020472.1		
slc38a7	Solute carrier family 38, member 7	Putative sodium-coupled neutral amino acid transporter 7	*D. rerio*	NM_001003648.1		
zgc:154088	zgc:154088	Putative sodium-coupled neutral amino acid transporter 9	*D. rerio*	NM_001079999.1		
slc38a10	Solute carrier family 38, member 10		*D. rerio*	XM_685145.4		
zgc:110221	zgc:110221	Putative sodium-coupled neutral amino acid transporter 11	*D. rerio*	NM_001017644.1		

[a] From www.bioparadigms.org.
[b] Na^+-coupled cotransport.
[c] Na^+-coupled cotransport and H^+-coupled exchange.

Table 10.40 The solute carrier family 39 (the metal ion transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc39a1	Solute carrier family 39 (zinc transporter), member 1	ZIP-1; solute carrier family 39 member 1; zinc transporter ZIP1; zrt- and Irt-like protein 1	*Danio rerio* *Takifugu rubripes* *Salmo salar* *Oncorhynchus mykiss*	NM_212583.2 NM_001032701.1 BT045888.1 BT045274.1 AY387045.1	Feeney et al., 2005; Qiu et al., 2005; Kambe et al., 2006; Zheng et al., 2008 Qiu et al., 2005 Qiu et al., 2007	Zn^{2+b}	Zn^{2+c}
frzip2p	ZIP zinc uptake transporter 2		*T. rubripes*	NM_001032674.1	Qiu and Hogstrand, 2005	Zn^{2+b}	Zn^{2+d}
slc39a3	Solute carrier family 39 (zinc transporter), member 3		*D. rerio*	NM_001080619.1	Feeney et al., 2005; Zheng et al., 2008	Unknown	–
slc39a4	Solute carrier family 39 (zinc transporter), member 4	ZIP4	*D. rerio*	NM_001130777.1	Feeney et al., 2005; Kambe et al., 2006	Zn^{2+b}	–
slc39a5	Solute carrier family 39 (zinc transporter), member 5	ZIP5	*D. rerio*	XM_685166.4	Kambe et al., 2006	Unknown	–
slc39a6	Solute carrier family 39 (zinc transporter), member 6	ZIP6; zinc transporter ZIP6	*D. rerio*	NM_001001591.1	Yamashita et al., 2004; Feeney et al., 2005; Kambe et al., 2006; Poulton et al., 2010	Unknown	–

(Continued)

Table 10.40 *(Continued)*

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc39a7	Solute carrier family 39 (zinc transporter), member 7	ZIP7; histidine-rich membrane protein Ke4; solute carrier family 39 member 7; zinc transporter Slc39a7	*D. rerio* *S. salar*	NM_130931.2 NM_001139771.1	Feeney et al., 2005; Kambe et al., 2006; Zheng et al., 2008; Hwang et al., 2009	Mn^{2+}[b]	
slc39a9	Solute carrier family 39 (zinc transporter), member 9	ZIP-9; ZIP9; solute carrier family 39 member 9; zinc transporter ZIP9; zrt- and Irt-like protein 9	*D. rerio* *Ictalurus punctatus*	NM_001013540.1 NM_001200431.1	Feeney et al., 2005; Kambe et al., 2006	Unknown	
slc39a10	Solute carrier family 39 (zinc transporter), member 10	ZIP-10; ZIP10; solute carrier family 39 member 10; zinc transporter ZIP10; zrt- and Irt-like protein 10	*D. rerio*	NM_200671.1	Feeney et al., 2005; Kambe et al., 2006; Zheng et al., 2008	Unknown	
slc39a11	Zinc transporter SLC39A11		*S. salar* *I. punctatus*	NM_001173736.1 NM_001200685.1		Unknown	
slc39a13	Solute carrier family 39 (zinc transporter), member 13	ZIP-13; solute carrier family 39 member 13; solute carrier family 39, member 13; zinc transporter ZIP13; zrt- and Irt-like protein 13	*D. rerio* *S. salar*	NM_001005306.3 NM_001173795.1	Feeney et al., 2005; Kambe et al., 2006	Zn^{2+}[b]	

[a] From www.bioparadigms.org.
[b] Unknown.
[c] Zebrafish.
[d] Fugu.

Table 10.41 The solute carrier family 40 (the basolateral iron transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
slc40a1	Solute carrier family 40 (iron-regulated transporter), member 1	FPN1; slc39a1; ferroportin-1; ferroportin1; iron-regulated transporter; solute carrier family 39 (iron-regulated transporter), member 1; solute carrier family 40 member 1; weh; weissherbst	*Danio rerio*	NM_131629.1	Ransom et al., 1996; Donovan et al., 2000; North and Zon, 2003; Eckfeldt et al., 2005; Fraenkel et al., 2005; Cooper et al., 2006; Davidson and Zon, 2004; De Domenico et al., 2007; Craig et al., 2009; Hong et al., 2010	Fe^{2+b}	Fe^{2+c}

[a] From www.bioparadigms.org.
[b] Facilitated transport (?).
[c] Zebrafish.

Table 10.42 The solute carrier family 41 (the MgtE-like magnesium transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	equence accession ID	Reference	Predominant substrates in vertebrates[a]
slc41a1	Solute carrier family 41, member 1	–	*Danio rerio*	XM_002663821.1	Wingert et al., 2007	Mg^{2+} (Sr^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, Cd^{2+})
slc41a2	Solute carrier family 41, member 2	–	*Salmo salar*	NM_001173587.1		Mg^{2+} (Ba^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+})

[a]From www.bioparadigms.org.

Table 10.43 The solute carrier family 42 (the Rh ammonium transporter family).

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]	Substrates tested in fish
rhag	Rhesus blood group-associated glycoprotein	Rh blood group-associated glycoprotein; ammonium transporter Rh type A	*Danio rerio* *Takifugu rubripes* *Gasterosteus aculeatus* *Oncorhynchus mykiss*	NM_212845.1 AB218979.1 DQ523516.1 EF667352.1	Huang and Peng, 2005; Nakada et al., 2007a, 2007b; Braun et al., 2009a, 2009b; Nawata et al., 2007	$NH4^{+}$,[b] (CO_2)	Methylammonium[c]
rh50	Rh50-like protein		*D. rerio* *T. rubripes*	NM_131547.1 AY116073.1 AY613959.1 AY116072.1	Liu et al., 2000; Huang and Peng, 2005		

rhbg	Rhesus blood group, B glycoprotein	Ammonium transporter Rh type B; rh family type B glycoprotein; rh type B glycoprotein; rhesus blood group family type B glycoprotein	*D. rerio* *O. mykiss* *Tetraodon nigroviridis* *T. rubripes* *Oryzias latipes* *Kryptolebias marmoratus* *G. aculeatus*	NM_200071.2 EF051113 EF051114 AY865614.1 AY116074.1 AY353247.1 DQ995211.1 DQ523519.1 DQ523517.1	Huang and Peng, 2005; Kikuta et al., 2007; Nakada et al., 2007a; Braun et al., 2009a, 2009b; Perry et al., 2010 Nawata et al., 2007; Nakada et al., 2007b; Hung et al., 2007	NH4+[b]	Methylammonium[c]
rhcg(1)	Rhesus blood group, C glycoprotein	Ammonium transporter Rh type C-like 2; drRhcg1; rh family type C glycoprotein-like 2; rh type C glycoprotein-like 2; rhesus blood group family type C glycoprotein-like 2	*D. rerio* *O. mykiss* *K. marmoratus* *T. rubripes* *T. nigroviridis*	NM_207082.1 DQ431244.1 EF051115 DQ995210.1 AY116075.1 AY865615.1	Huang and Peng, 2005; Nakada et al., 2007a; Braun et al., 2009a; Nawata et al., 2007; Hung et al., 2007; Nakada et al., 2007b	NH_4^{+b}	Methylammonium[c]
rhcg(2)	Rhesus blood group, C glycoprotein	Ammonium transporter Rh type C-like 2; drRhcg1; rh family type C glycoprotein-like 2; rh type C glycoprotein-like 2; rhesus blood group family type C glycoprotein-like 2	*O. mykiss* *K. marmoratus* *T. rubripes* *O. latipes* *G. aculeatus*	AY619986.1 DQ423779.1 AY116076.1 AB036511.1 ABF69690.1	Huang and Peng, 2005; Hung et al., 2007; Nakada et al., 2007b; Kitano and Saitou, 2000; Wu et al., 2010		Methylammonium[c]
rhd	Rh blood group, D antigen	Rh30-like protein	*D. rerio* *O. mykiss* *Salmo salar* *T. rubripes* *T. nigroviridis*	NM_001024819.1 AY207445.1 EF062577.1 EF062578.1 AY340237.1 AY116077.2 AY865612.1	Nakada et al., 2007a; Huang and Peng, 2005; Nawata et al., 2007		

[a]From www.bioparadigms.org.
[b]H^+-coupled exchange.
[c]Fugu.

Table 10.44 The solute carrier family 43 (the Na^+-independent, system-L like amino acid transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc43a1a	Solute carrier family 43, member 1a	Solute carrier family 43, member 1	*Danio rerio*	NM_199897.1	Yang et al., 2007; Sekine et al., 2009	Neutral amino acids[b]
slc43a1b	Solute carrier family 43, member 1b	Large neutral amino acids transporter small subunit 3	*D. rerio*	NM_001083000.1		Neutral amino acids[b]
slc42a2a	Solute carrier family 43, member 2a	Solute carrier family 43, member 2	*D. rerio*	NM_200251.1	Gomez et al., 2009	Neutral amino acids[b]
slc43a2b	Solute carrier family 43, member 2b	Large neutral amino acids transporter small subunit 4	*D. rerio*	NM_001008585.1		Neutral amino acids[b]

[a]From www.bioparadigms.org.
[b]Facilitated transport.

Table 10.45 The solute carrier family 44 (the choline-like transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc44a1	Solute carrier family 44, member 1	cdw92	*Danio rerio*	XM_002660581.1		Choline
slc44a2	Solute carrier family 44, member 2	Choline transporter-like protein 2; solute carrier family 44 member 2	*D. rerio* *Salmo salar*	XM_691494.3 NM_001140367.1		–
slc44a4	Solute carrier family 44, member 4	Choline transporter-like protein 4	*D. rerio*	NM_200413.1	Jima et al., 2009	–
slc44a5a	Solute carrier family 44, member 5a	Choline transporter-like protein 5-A; slc44a5; solute carrier family 44 member 5-A	*D. rerio*	XM_691470.4		–
slc44a5b	Solute carrier family 44, member 5b	Choline transporter-like protein 5-B; solute carrier family 44 member 5-B; solute carrier family 44, member 5	*D. rerio*	XM_681327.4		–

[a] From www.bioparadigms.org.

Table 10.46 The solute carrier family 45 (the putative sugar transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates
slc45a2	Solute carrier family 45, member 2	Absent in melanoma 1; membrane-associated transporter protein	*Danio rerio* *Oryzias latipes*	NM_001110377.1 NM_001104758.1	Braasch et al., 2007; Fukamachi et al., 2001, 2008	–
slc45a3	Solute carrier family 45, member 3		*D. rerio*	NM_001098195.1		–
slc45a4	Solute carrier family 45, member 4		*D. rerio*	XM_680834.4		–

Table 10.47 The solute carrier family 46 (the folate transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc46a1	Solute carrier family 46, member 1	PCFT/HCP1; heme carrier protein 1; proton-coupled folate transporter	*Danio rerio*	NM_200285.1		Folate[b]
slc46a3	Solute carrier family 46, member 3		*D. rerio*	XM_679046.2		–

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport.

Table 10.48 The solute carrier family 47 (the multidrug and toxin extrusion (mate) family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc47a1	Solute carrier family 47, member 1		*Danio rerio*	NM_001014310.2		Organic cations[b]
slc47a2	Solute carrier family 47, member 2	MATE-1; multidrug and toxin extrusion protein 1; solute carrier family 47 member 1	*D. rerio*	NM_001080179.1		Organic cations[b]

[a] From www.bioparadigms.org.
[b] H^+-coupled exchange.

Table 10.49 The solute carrier family 48 (the heme transporter family). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc48a1a	oLute carrier family 48 (heme transporter), member 1a	HRG-1B; heme transporter hrg1-B; heme-responsive gene 1 protein homolog B; solute carrier family 48 member 1-A	*Danio rerio*	NM_001002424.1		Heme[b]
slc48a1b	Solute carrier family 48 (heme transporter), member 1b	HRG-1A; heme responsive gene 1; heme transporter hrg1-A; heme-responsive gene 1 protein homolog A; hrg-1; solute carrier family 48 member 1-B; zHRG-1	*D. rerio*	NM_200006.1	Song et al., 2004; Rajagopal et al., 2008	Heme[b]

[a] From www.bioparadigms.org.
[b] H^+-coupled cotransport (?).

Table 10.50 The solute carrier family 49 (transporters of the major facilitator superfamily). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
flvcr1	Feline leukemia virus subgroup C cellular receptor 1	Feline leukemia virus subgroup C receptor-related protein 1; solute carrier family 49 member 1	*Danio rerio*	NM_001025522.1	–	Heme and proximal planar porphyrins[b]
mfsd7	Major facilitator superfamily domain containing 7	Major facilitator superfamily domain-containing protein 7; solute carrier family 49 member 2	*D. rerio*	NM_001020587.1	–	Heme[b]
dirc2	Disrupted in renal carcinoma 2	Solute carrier family 49 member 4	*D. rerio*	NM_001004597.1	–	

[a] From www.bioparadigms.org.
[b] Unknown.

Table 10.51 The solute carrier family 50 (sugar efflux transporters). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
slc50a1	Solute carrier family 50 (sugar transporter), member 1	RAG1-activating protein 1 homolog; recombination activating gene 1 activating protein 1	*Danio rerio*	NM_001012497.1	–	Glucose[b]

[a] From www.bioparadigms.org.
[b] Facilitated transport.

Table 10.52 The solute carrier family 51 (transporters of steroid-derived molecules). No substrates have been tested in fish species.

Official symbol	Name	Other designations	Species	Sequence accession ID	Reference	Predominant substrates in vertebrates[a]
zgc:92111	zgc:92111	OST-alpha; organic solute transporter alpha-like; organic solute transporter subunit alpha	*Danio rerio*	NM_001004546.1		Glycine and taurine conjugated bile acid species[b]

[a]From www.bioparadigms.org.
[b]Facilitated transport.

slc14, with 3 members, is involved in the transport of urea; slc18, with 4 members, transports acetylcholine (by the vesicular slc18a3-type acetylcholine transporter) and biogenic amines (by the vesicular slc18a1- and slc18a2-type monoamine transporters) into secretory vesicles, which are then discharged into the extracellular space by exocytosis; slc22, with 8 members, functions in endogenous organic cation/anion/zwitterion (e.g., carnitine, betaines, amino acids) transport and thus is very important in drug transporter functions; slc42, with 6 members that appear to be involved in $NH4^+$ transport (whereas their gene names are rhag, rhbg, and rhcg, etc.); and slc44, with 5 members, appears to be involved in choline transport.

Transport of Vitamins and Cofactors

Four families participate in vitamin or cofactor transport (Table 10.2; see also Tables 10.21, 10.25, 10.34, 10.47, and literature cited therein): slc19, with 1 member found so far in teleost fish, transports folate and thiamine, energised by a trans-membrane H^+/OH^- gradient; slc23, with 2 members, transports ascorbic acid; slc33, with a single member, is an acetyl coenzyme A transporter; and slc46, with 2 members, is involved in H^+-coupled folic acid transport.

Nucleoside/Nucleotide Transport

Three families carry out the transport of nucleosides and nucleotides (Table 10.2; see also Tables 10.30, 10.36, and literature cited therein): slc28, with no members found so far in teleost fish, functions in Na^+-coupled nucleoside transport; slc29, with 1 member, mediates (along with the slc28 transporters) uptake of natural nucleosides (among them adenosine); and slc35, with 22 members, transports nucleotide sugars (pooled in the cytosol) into the lumen of the Golgi apparatus and endoplasmic reticulum, wherein occurs most of the synthesis of glycoconjugates.

Transport of Fatty Acids, Prostaglandins, and Steroid Sulfates

One SLC family is mainly involved in transport of fatty acids, prostaglandins, and steroid sulfates (Table 10.2; see also Table 10.29 and literature cited therein): slc27, with 5 members found so far in teleost fish, participates in the transport of long-chain fatty acids. In addition, slco2-type slc21 transporters, with 3 members, function in prostaglandin and steroid sulphate transport (refer to the following text).

Transport of Organic Anions, Prostaglandins and Steroid Sulfates, and Thyroid Hormones

One SLC family, divided into several subfamilies (slco sub-families) is specifically involved in organic anion transport (Table 10.2; see also Table 10.23, and literature cited therein): slc21, with 14 members found so far in teleost fish, participates in the transport of organic anions, drugs, prostaglandins, steroid sulfates, and thyroid hormones. Most of the slc21 proteins have not yet been well characterized.

Heme Transport

Two SLC families mediate the transport of heme (Table 10.2; see also Tables 10.49 and 10.50): slc48, with 2 members found so far in teleost fish, and slc49, with 3 members, seem to participate in the transport of heme.

Transport across Mitochondrial Membranes

One SLC family includes four subfamilies of mitochondrial transporters (Table 10.2; see also Table 10.27 and literature cited therein): slc25, with 46 members, comprises the mitochondrial carriers that shuttle a variety of

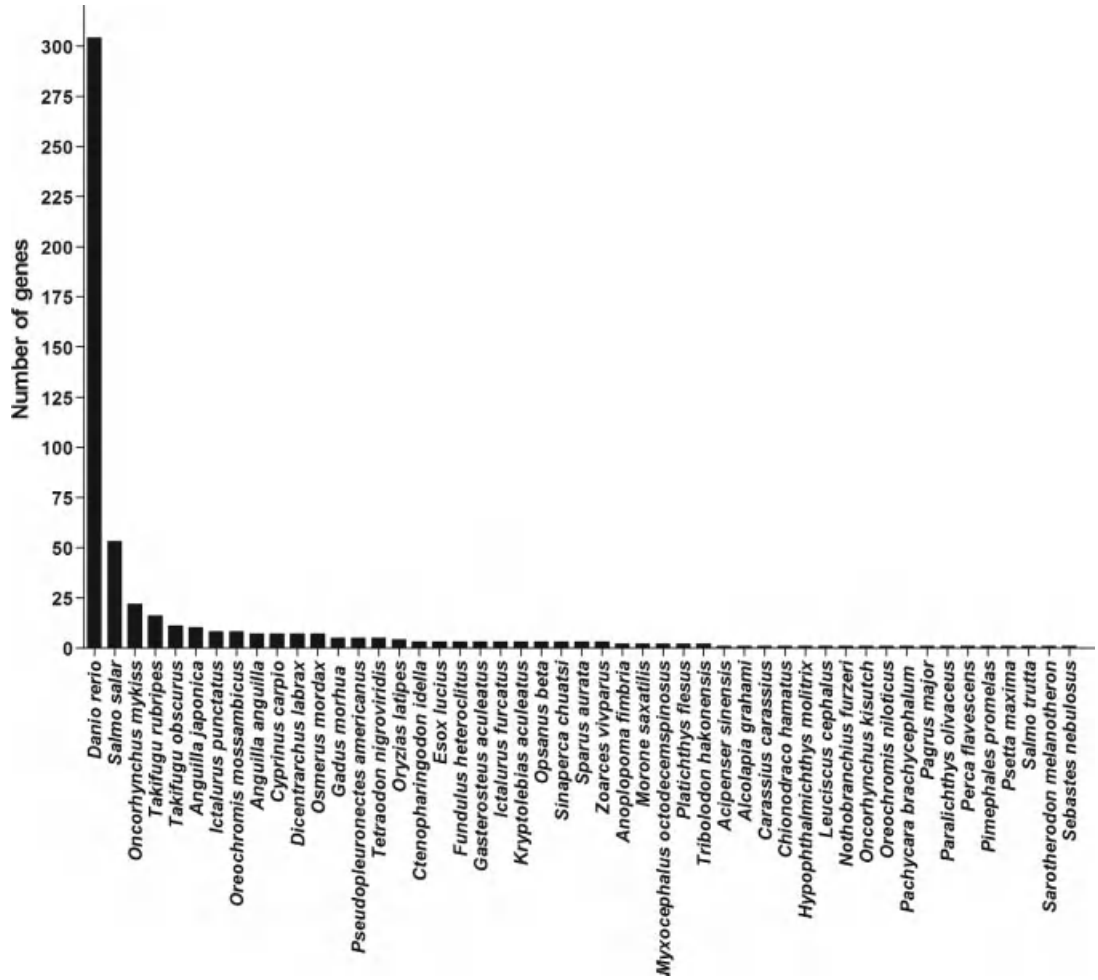

Figure 10.1 Genes of SLC members from teleost fish species, as obtained by GenBank database consulting. A 3-month survey has been conducted between December 2010 and March 2011.

metabolites across the mitochondrial inner membrane; ucp1, ucp2, and ucp3, with 5 members, work as uncoupling proteins or proton pumps involved in mitochondrial energetics.

Function in Teleost Fish SLC Transporters

Only a limited number of teleost fish genes has been functionally characterized, i.e., the cloned transporter has been tested for transport function by expression in a suitable heterologous expression system (Bossi et al., 2007; Markovich, 2008; Musa-Aziz et al., 2010). Only this way, the functional properties, in terms of kinetic parameters, substrate specificities, and/or inhibition patterns of the expressed transport proteins can unequivocally be assessed. To our knowledge, only 41 teleost fish transporters have been tested in vitro using such heterologous systems (Table 10.2). Fifteen are involved in inorganic cation/anion transport, two in amino acid/oligopeptide transport, six in

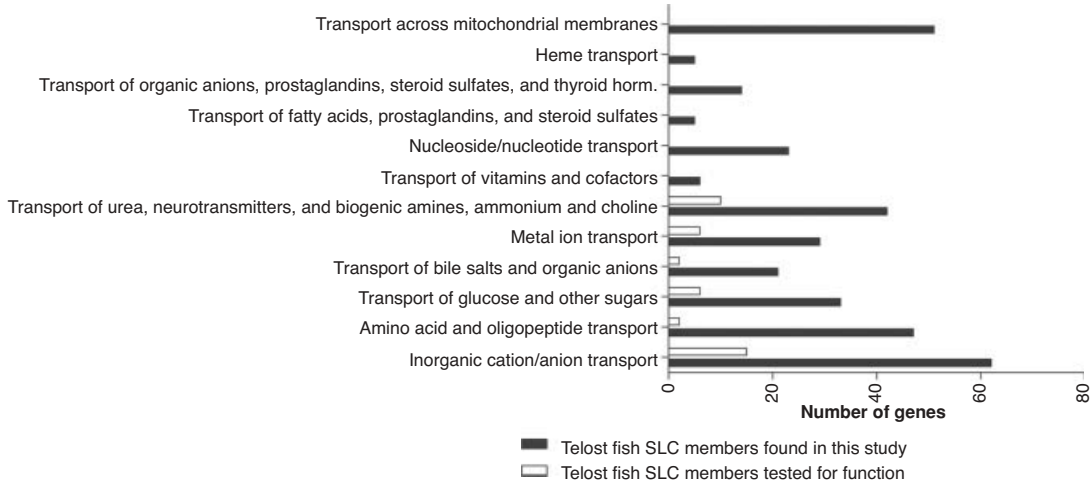

Figure 10.2 A comparison between the number of teleost fish members found in this study and the number of teleost fish SLC members that have positively been tested for function.

transport of glucose and other sugars, six in metal ion transport, two in transport of bile salts and organic anions, ten in transport of urea, neurotransmitters and biogenic amines, ammonium, and choline. No functional tests have been performed so far on SLC members involved in the transport of vitamins and cofactors, nucleosides/nucleotides, fatty acids, prostaglandins, steroid sulfates, organic anions, thyroid hormones, and heme. These general observations open the question on the necessity of focusing on these undisclosed transporters, with special regard to those that mediate the transport of vitamins, nucleosides, and nucleotides, major topics in cultured fish research and applications.

Most of the functional studies have been performed in order to enlighten the role(s) of the transporters in the fundamental processes that allow teleost fish to live in waters at different salinities (e.g., cation, anion, urea, ammonium transporters) and different oxygen levels (glucose and other sugar transporters) (see Figure 10.2 and Table 10.2; for details, see Tables 10.3–10.52). Studies have also been conducted to establish how the uptake of biologically relevant (and/or toxic) metal ions (among which iron, copper, cadmium ions) may affect fish embryo development. It is a matter of fact that at the moment very limited information is available on the transport events that mediate the uptake of amino acids and oligopeptides, in spite of the physiological relevance of such nutrients intestinal absorption in fish growth (mostly in cultured species). However, when studied in detail, each selected transporter has often been cloned from more than one species, in order to compare the function in a variety of teleost fish background (Figure 10.3). As shown in Figure 10.3, zebrafish seems to be the most popular teleost fish model for functional studies, followed by rainbow trout and winter flounder (*Pseudopleuronectes americanus*). In particular, due to the good annotation of its genes and genome, zebrafish is often used to extract from the database good full-length sequences that are useful (1) to generate/recruit adequate molecular tools for functional analysis in the zebrafish itself; and (2) to generate/design molecular tools to jump to another fish species.

Conclusions

In teleost fish, the SLC gene series comprises 50 families and 338 members. These transport

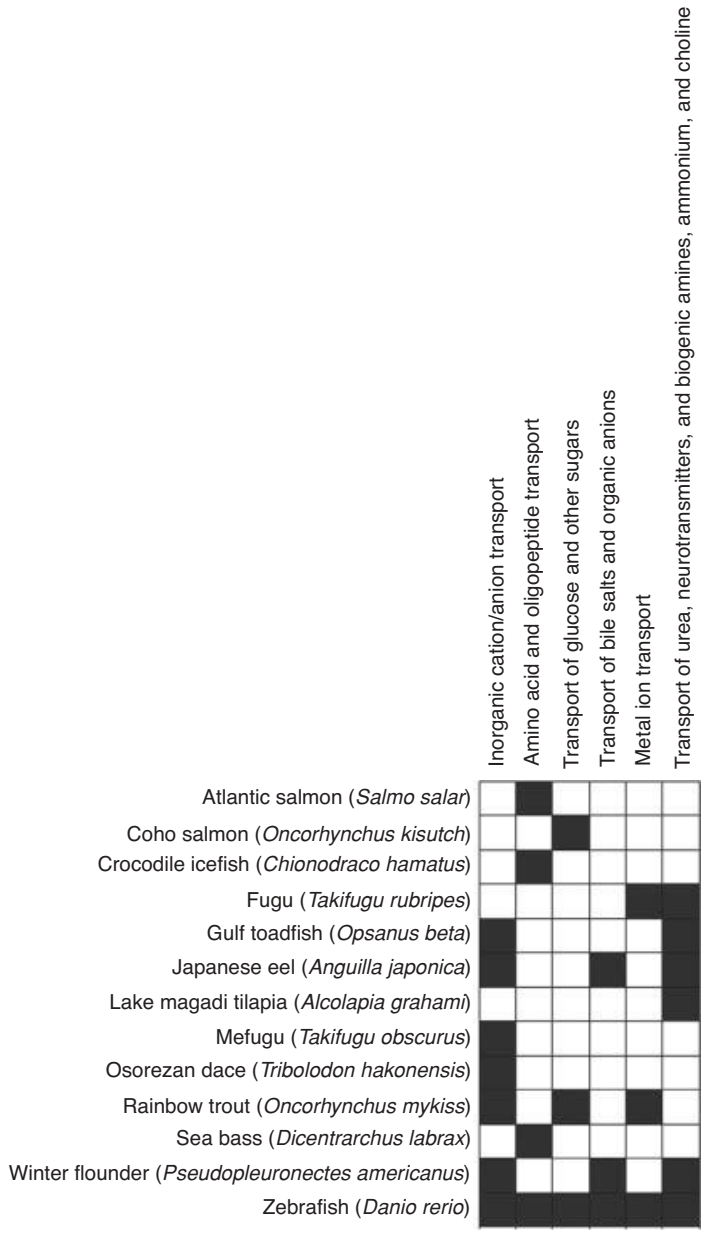

Figure 10.3 Functional expression of teleost fish SLC members. The teleost fish models whose cloned transporters have been subjected to functional analyses in heterologous expression systems are indicated.

proteins mediate the movement of virtually all soluble molecules across cell membranes. In 2004, classification of symporters, antiporters, and passive transporters into the human SLC families helped to rationalize the studies and the efforts, which led to the comprehension of the basic mechanisms that mediate the transport of both known

and novel substrates across membranes, and contributed a "little revolution" in human physiology, medicine, genetics, pharmacology, and cancer chemotherapy. In the last ten years, by combining bioinformatics, computational biology, genome/gene/cDNA/EST sequencing, and functional genomics, a large amount of sequence information has been generated for teleost fish. With a few exceptions, the largest part of this information is available to the scientific community. However, teleost fish sequences often lack adequate support annotation so that it is not always easy to decide which sequence is the one of interest, mostly in the presence of redundancy in databanks and/or duplicated genes. Today, a functional approach to sequences of genes, mRNAs, and proteins involved in solute transport functions across teleost fish cell membranes is needed to elucidate the role of transporters in teleost fish physiology, in the perspective of confirming old and establishing new concepts, and applying the most relevant information to health, nutrition, and welfare of cultured species.

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Chapter 11
Next-Generation Sequencing and Functional Genomic Analysis in Rainbow Trout

Mohamed Salem

Abstract: Recent advances in sequencing technology allowed development of several genomic tools for rainbow trout, including fast, highly efficient, and low-cost characterization of the transcriptome; in addition, it will soon provide a complete draft of the genome. Further, large-scale single nucleotide polymorphism (SNP) identification has been accomplished using deep sequencing of the transcriptome, reduced representation, and restricted site-associated DNA (RAD) libraries of the genome. An SNP consortium has been organized to create a rainbow trout high-throughput SNP platform. RNA-Seq technique has been used to identify SNP markers predictive of production traits. Consequently, modern sequencing technologies brought enormous potentials for development of informative and precise functional genomics tools that are vital for the growth of rainbow trout aquaculture. Application of the state-of-the-art high-throughput SNP chips technologies in a genome-wide approach will allow identification of genes/QTLs that regulate production traits.

Introduction

Recent developments of the next-generation sequencing (NGS) technologies have permitted fast, accurate, and reasonably priced broad range of genomic applications including sequencing genomes, sequencing transcriptomes, and sequencing noncoding RNAs; gene expression studies; and discovery of single nucleotide polymorphisms (SNPs) and other forms of allelic variations. New sequencing technologies (both the second-generation and the third-generation sequencing technologies) will have major impacts on animal and plant genetic breeding programs, allowing replacement of the traditional single-gene/QTL approach with a holistic whole-genome selection methodology in dealing with aquaculture-related functions such as performance and production traits. In this chapter, I will introduce the principles of several most commonly used NGS platforms and their potential applications for aquaculture functional genomic analysis.

Next-Generation Sequencing Technology

Currently, the three most widely used DNA sequencing technologies are provided by the 454 Roche Applied Science, Illumina, and the Applied Biosystems (Karow and Toner, 2011). Each technology utilizes a complex interaction of sequencing steps, chemistry, hardware, and software. Table 11.1 shows

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

Table 11.1 Comparison of commonly used NGS platforms from Roche, Illumina, and AB/SOLiD. The table is based on information available from NGS technology providers in early 2011 (Roche-gsjunior, Roche-454, Illumina-systems/ga, Illumina-systems/hiseq, AB/SOLiD, AB/SOLiD-5500 xl).

Platform	Read length bp (quality)	Output Gb/run	Output M read/run	Run time (days)	Lanes/ flow cell
Roche/454's GS Junior	400, $q > 20$	0.035	0.1	0.5	12
Roche/454's GS FLX Titanium	400, $q > 20$	0.4–0.6	1	0.5	12
Illumina Genome Analyzer IIx	35–2 × 150, $q > 30$	10–95	640 paired-end	2–14	8
Illumina HiSeq 2000	35–2 × 100, $q > 30$	26–200	1000–2000 paired-end	1.5–8	8
AB/SOLiD™ 5500 System	75–2 × 60, 99.99%	90	1400	7	6
AB/SOLiD™ 5500 xl System	75–2 × 60, 99.99%	180–300	2800–4800	7	12

the comparison of throughput, read length, and quality of these platforms based on information available from the NGS technology providers (Roche-gsjunior, Roche-454, Illumina-systems/ga, Illumina-systems/hiseq, AB/SOLiD, AB/SOLiD-5500xl). With these NGS technologies, libraries are constructed and sequenced from amplified fragments of single-stranded DNA. The most significant advantage of NGS technology is saving the time-consuming, laborious, and equipment-associated steps of cloning and colony picking of the Sanger-based capillary sequencing. The second most important advantage of NGS is the tremendous increase in sequencing output in terms of the number of reads (1–4800 million reads) and total bases per run (0.4–300 Gbp) compared with 96 reads of 750–1000 bp each in the capillary-based sequencing. The most problematic compromise of NGS is the relatively shorter read length of 35–400 bp as compared with 750–1000 bp of the capillary-based sequencing. Discussions on this chapter will mainly focus on the 454 Roche/pyrosequencing and the Illumina sequencing technologies that have been used in rainbow trout studies. Detailed description of NGS technologies can be found in the literature (Mardis, 2008b, 2008a; Shendure and Ji, 2008; Zhou et al., 2010; Kircher and Kelso, 2010).

454 Roche/Pyrosequencing

Sequencing workflow of the 454 Roche GS FLX and GS Junior platforms consists of the following basic steps (Figure 11.1A). Double-stranded DNA (genomic DNA, cDNA, or PCR) is fractionated by nebulization into 300–800 bp fragments. Fractionation of short DNA samples, for example, miRNA is not necessary. Nebulized DNA fragments are ligated to 2 titanium adaptors A/B specific for the 3′ and 5′ ends. These adaptors are used for amplification, purification, and sequencing. Single-stranded DNA fragments of the library are fixed to specially designed beads so that one DNA fragment is attached to one bead. Bead-immobilized fragments and PCR reagents are emulsified into water-in-oil microreactors. DNA is amplified inside these microreactors (emPCR), producing millions of copies of each fragment per bead. Bead-bound fragments are released from microreactors and enriched for next liquid handling workstations. Each fragment-bound bead is loaded onto a

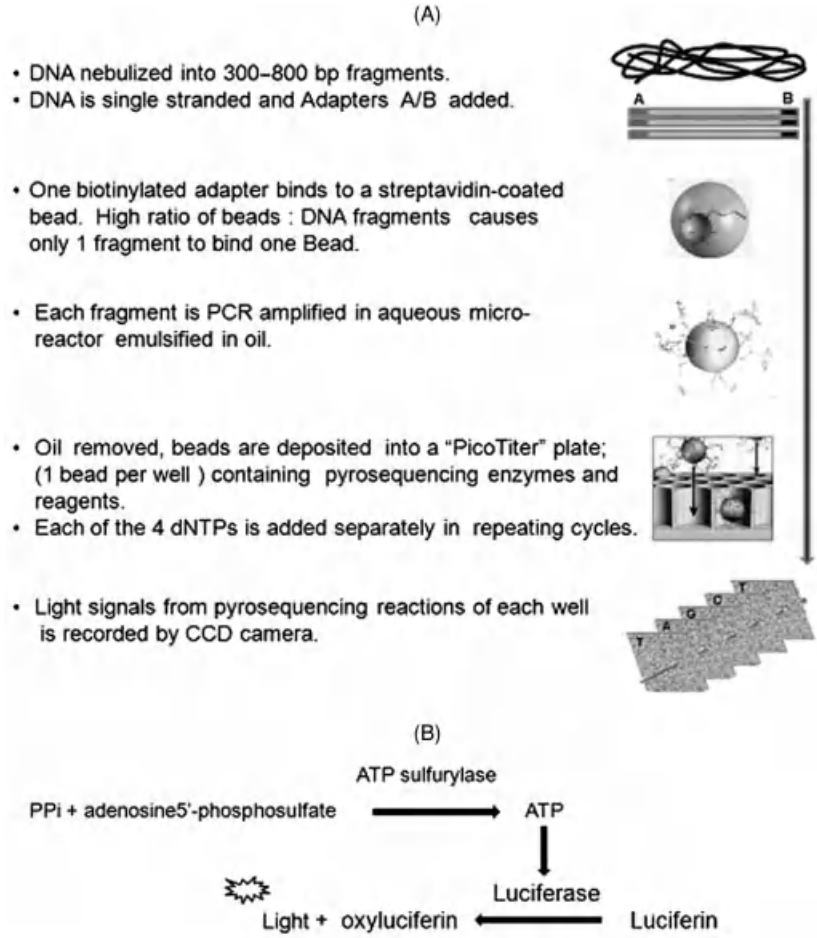

Figure 11.1 (A) Pyrosequencing workflow of the 454 Roche GS FLX and GS Junior platforms. (B) Pyrosequencing chemistry following addition of dNTPs to free 3′ end of the DNA fragment. See color insert.

single well on a PicoTiterPlate for sequencing. The diameter of the PicoTiterPlate well permits for only one bead per well. The well also contains all requirements of sequencing reactions, such as DNA polymerase, ATP sulfurylase, and luciferase. Hundreds of thousands of fragments (each enclosed in one well) are sequenced in parallel by the so-called sequencing-by-synthesis. Nucleotides are added in a fixed order to complement template strands. A dNTP is attached to the free 3′ end of the fragment and a pyrophosphate (PPi) group is released from the reaction. ATP sulfurylase enzyme uses PPi and adenosine 5′-phosphosulfate to make a molecule of ATP. Luciferase enzyme uses ATP to convert luciferin substrate to oxyluciferin (Figure 11.1B). Each addition of a nucleotide results in a chemiluminescent signal that is recorded by the CCD camera. A combination of signal intensities and positions across the PicoTiterPlate are analyzed by specific software to determine the sequence of each fragment. About 0.1–1 million reads (with the GS Junior System and the GS FLX System, respectively) are produced per run in an approximately 10-hour run time. The read length at sequence

quality phred $q \geq 20$ (<0.01% sequence error) is 400 bp.

While this book was under review, a newer GS FLX+ System with read lengths up to 1 kb was released, allowing more power of de novo sequencing of large and complex organisms. Bioinformatics software packages are available for de novo assembly, mapping sequence data to a known reference sequence with sequence variant detection.

Illumina Sequencing

Illumina's sequencing platform employs a robust four-color sequencing-by-synthesis technology. Illumina Genome Analyzer technology is built upon the idea of the reversible terminator-based chemistry that is similar to that of the conventional Sanger sequencing. A fluorescently labeled base is added and imaged each sequencing cycle, and reaction is paused after each base addition. The fluorescently labeled terminator is cleaved to allow next base incorporation. The base-by-base sequencing enables accurate data suitable for a wide range of applications.

Similar to 454 Roche/Pyrosequencing, sample preparation involves DNA shearing using different methods, for example, a nebulizer, DNA end polishing, and ligation of two adapters to the 3′ and 5′ ends. Fragments of size range of 150–200 bp are gel extracted and PCR amplified. Next step is cluster generation where single DNA fragments are immobilized by hybridization onto oligonucleotide-coated flow cell; fragments are amplified to form flow cell-bound clusters. Each cluster consists of several identical copies of the original template fragment. After cluster generation, the flow cell is loaded into the Genome Analyzer and subjected to repeated rounds of massively parallel sequencing, simultaneously sequencing millions of clusters to generate billions of nucleotide bases from a single run. The Genome Analyzer computer tracks the run, processes images, and base calling (Figure 11.2).

The Illumina's platform supports both single read and paired-end sequencing that is suitable for a wide range of applications. Long-insert paired-end reads allows efficient genome sequence assembly, de novo sequencing, and detection of chromosomal structural variation. On the other hand, short-insert paired-end capability enables high-resolution re-sequencing, transcriptome analysis (RAN-Seq) and miRNA discovery.

Comparison of Illumina Sequencing and 454 Pyrosequencing

Both Illumina and 454 Roche/Pyrosequencing have been used in different rainbow trout genome projects, which showed the pros and cons of each technology. The main differences are read length and throughput: Illumina sequencing generates relatively short sequence reads of approximately 100 bp, but with enormously high throughput of approximately 20 Gb; in contrast, 454 sequencing generates relatively long reads of approximately 400 bp, but its throughput is only approximately 0.4 Gb. Technologically, 454 Roche/Pyrosequencing is more suitable for de novo characterization of nonmodel genomes/transcriptomes, and Illumina is more suitable for re-sequencing known genomes, digital gene expression, and miRNA discovery. However, such technological capability difference is complicated by the cost associated with the sequencing platforms. For instance, Illumina HiSeq 2000 instrument can generate 20 Gb sequence per sequencing lane that cost only $3000–4000, while one run of 454 sequencing can only generate 400 million bp that cost over $10,000. As a result, many researchers are using Illumina's paired-end reads that allow greater ability to overcome characterizations of repetitive genomic regions and high complexity rearrangement. Ongoing characterization of the rainbow trout transcriptome using Illumina and 454 Roche/Pyrosequencing showed that a combination of the two

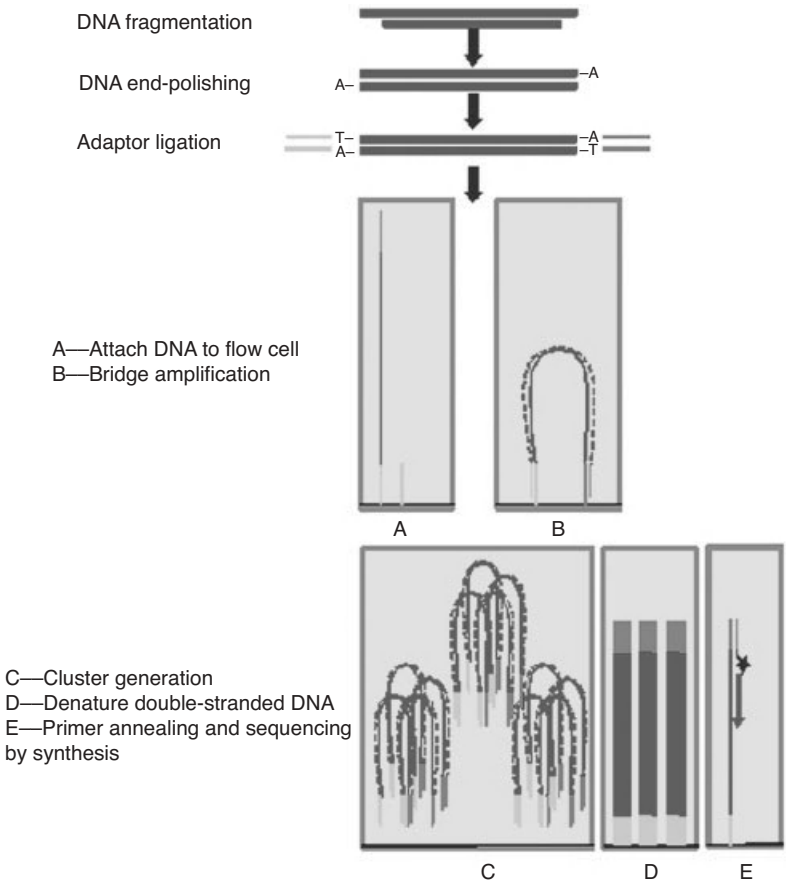

Figure 11.2 Illumina's sequencing-by-synthesis technology. Library preparation involves DNA fragmentation followed by end polishing and adaptor ligation. DNA fragments are immobilized onto flow cell and bridge amplified to form clusters. After cluster generation, the flow cell is loaded into the Genome Analyzer and subjected to repeated rounds of massively parallel sequencing-by-synthesis. See color insert.

technologies provides a more complete, cost-effective analysis (data not shown). Likewise, assemblies generated using Sanger and pyrosequencing data provide some advantage compared with assemblies generated using each data set alone (Salem et al., 2010). Therefore, when selecting the right technology or combination of technologies, it is important to take into account the aim of the project and the genomic information available for the organism. Data throughput versus long read length is the main parameter that influences the usefulness of technology for projects.

Applications of Next-generation Sequencing in Rainbow Trout

Aquaculture is the world's fastest growing agri-food business. With unsustainable high rates of human population growth and depletion of the natural fisheries resources, aquaculture is expected to continue to grow. Rainbow trout is among the most widely distributed and cultured fish species in many countries throughout the world including Europe, North America, Chile, Japan, and Australia. Rainbow trout attract great interest because of its

economic impacts; it is cultured both for food fish and for recreational fisheries. In addition, rainbow trout serves as an experimental model for research needed on closely related and economically important salmonid species such as Atlantic salmon and Pacific salmon and char (Thorgaard et al., 2002). On the other hand, rainbow trout is one of the most thoroughly studied fishes in many research areas, including carcinogenesis, toxicology, comparative immunology, disease ecology, physiology, and nutrition. For decades, rainbow trout has been used as a research model; more is known about the biology of rainbow trout than any other fish species (Thorgaard et al., 2002). However, a major constraint to increasing rainbow trout production efficiency is the lack of genetically improved strains for aquaculture (Gjerde, 2006, 2008). The large interest in rainbow trout for aquaculture and as a biomedical research model has led to development of several rainbow trout genomic resources (Davidson et al., 2006; Koop et al., 2008). Recently, NGS has become a rapid and cost-effective method for sequencing. Here, I will discuss some of the recent applications and future perspectives of the high-throughput NGS technologies in rainbow trout aquaculture. The progressively increasing depth and coverage of the NGS provide a very suitable method for de novo genome and transcriptome sequencing and for genetic marker development in nonmodel species such as rainbow trout.

Application in Sequencing the Rainbow Trout Genome

Full genome sequencing of several aquaculture species is now in process, including Atlantic salmon, catfish, cod, rainbow trout, blue fin tuna, and oyster (Johansen et al., 2009; Davidson et al., 2010; Rise and Quiniou, 2011). Sequencing the genome is an important step toward functional analyses in rainbow trout, including producing a high-throughput SNP genotyping platform for genomic analysis, fine mapping of QTL, whole genome association studies, genome-wide selection for improved aquaculture production traits, and genetic analysis of wild populations for fisheries management. Sequencing a draft reference genome for rainbow trout has been recently initiated; a consortium involves collaborators from USDA National Cold and Cool Water Aquaculture (USDA-NCCCWA), Washington State University, University of Oregon, University of California Davis, and West Virginia University (Palti et al., 2011). The project will produce a genome map and a draft reference genome sequence using the Illumina platform by sequencing the bacterial artificial chromosomes (BACs) from the physical map minimal tiling path (Palti, 2010; Miller et al., 2011). The minimal tiling path approach has been successfully used in other species, including *Caenorhabditis elegans*, *Arabidopsis thaliana,* and sequencing the human genome and individual human chromosomes (Coulson et al., 1986; Venter et al., 1996; Bentley et al., 2001; McPherson et al., 2001). A physical map of the rainbow trout has been constructed from fingerprinted BAC libraries (Palti et al., 2009). A minimum tiling path BAC clones were selected for paired-end Illumina sequencing. Assembly and annotation of these sequences will provide a complete draft of rainbow trout genome. The minimal tilling path strategy used in sequencing the rainbow trout genome is outlined in Figure 11.3.

In addition, there are two de novo genome sequencing projects being carried out independently by (1) Genoscope (whole genome sequence with 454 and Sanger BAC ends) and (2) West Virginia University (a genome survey project with 454 and Illumina). In phase I of the project at West Virginia University, a 2.3 X genome sequence coverage was obtained using the de novo whole genome shotgun sequencing on a 454 GS FLX long-read pyrosequencing platform. In phase II, more sequence coverage of the genome will be added. Sequences will be anchored/mapped to reference sequences from model fish species to improve assembly and for annotation and comparative mapping.

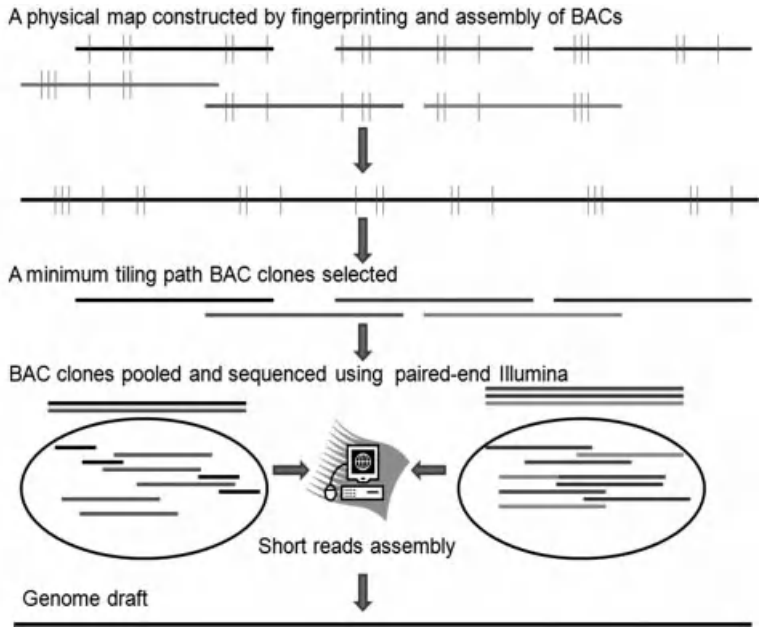

Figure 11.3 Application of NGS technology in sequencing the rainbow trout genome. BAC clones that constitute a minimal tilling path of the rainbow trout physical map are selected and pooled for sequencing using Illumina technology. Short reads from each pool are assembled into contigs. Contigs are aligned and joined together in a scaffolding process. A complete genome is drafted by positioning scaffolds along the physical map of the chromosomes. See color insert.

Application in Sequencing the Rainbow Trout Transcriptome

Transcriptome sequencing is important functional genomics analysis for gene discovery, genetic marker development, gene expression studies, SNP discovery, QTL testing, and genome scans of diversity and genomic selection (Vera et al., 2008). High-throughput deep sequencing of the rainbow trout transcriptome using 454-pyrosequencing technology produced 447 million bases, about 1.3 million reads with an average length of 344 bp. De novo assembly of the sequences yielded a total of 376,238 unique sequences, 151,847 tentative consensus sequences (average length of 662 bp), and 224,391 singletons (Figure 11.4; Salem et al., 2010). This study demonstrated the efficiency of the NGS for transcriptome analysis: A single pyrosequencing study yielded a large quantity of sequence data, more than all Sanger-based rainbow trout sequence data that have been produced by many laboratories over several years. A total of 1,416,404 ESTs, 447 million bases were obtained from a single 454-pyrosequencing run compared with 258,973 ESTs, 47 million bases obtained from Sanger-based sequences (Rainbow Trout Gene Index database release 7.0; Figure 11.4; Salem et al., 2010). The pyrosequencing identified a wealth of new genes. Subtracting the preexisting Sanger-based sequences from the pyrosequencing sequence data identified 282,357 gene transcripts.

Difficulty of assembling the relatively short reads of the NGS is a major concern when compared with Sanger-based sequence data (Ronaghi et al., 1998; Kristiansson et al., 2009). The pyrosequencing technology produces longer read lengths compared with

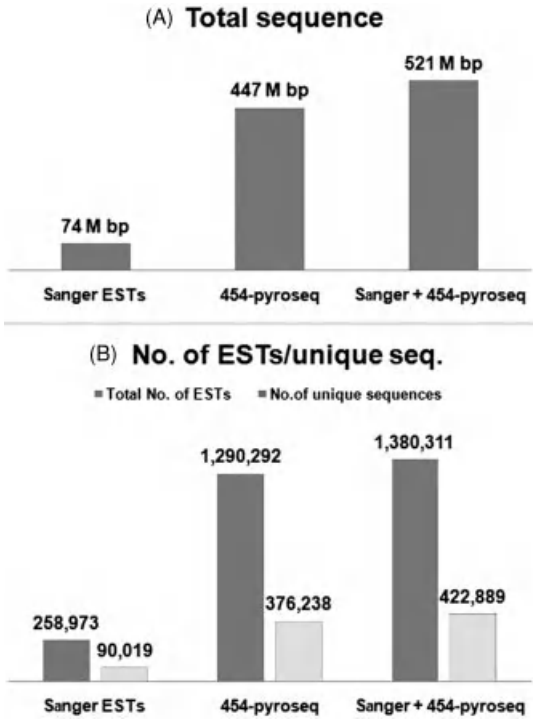

Figure 11.4 Relative contribution of the Sanger-based versus 454-pyrosequencing technologies to a rainbow trout transcriptome sequence: (A) total sequence yield and (B) number of ESTs.

other NGS technologies, which makes this technology suitable for rapid de novo transcriptome characterization of nonmodel species such as aquaculture species (Quinn et al., 2008; Vera et al., 2008). Relatively long-reads of the 454/GS-titanium sequencing Roche series allowed successful assembly of the rainbow trout pyrosequencing data. Assemblies generated with a hybrid sequencing approach, Sanger, and pyrosequencing provides some advantage when compared with assemblies generated using each data set alone. While the pyrosequencing data provided increased coverage and depth of the trout transcriptome, the Sanger-based data enhanced assembly of the short pyrosequence reads (up to 700 bp contigs length) but did not change the overall assembly percentage of pyrosequences. The average length of the pyrosequencing contigs was 662 bp, compared with 999 bp of the Sanger-based data and 785 bp of the combination assembly. The percentage of the assembled pyrosequencing reads was 83% compared with 81% of both the Sanger-based and the combination assemblies.

Rainbow trout is a member of the family Salmonidae, believed to be partially tetraploids because of whole genome duplication that occurred 25–100 mya (Allendorf and Thorgaard, 1984; Palti et al., 2004). Some studies showed duplicate gene expression of half of the protein-coding loci in salmonids (Bailey et al., 1978). Duplicate genes in the rainbow trout genome cause difficulties in assembly and annotation of the EST sequences and lead to identification of false SNPs (Sanchez et al., 2009). Even with very stringent assembly parameters, paralogous sequences (from different loci in genome) can be assembled into one sequence and false SNPs cannot be distinguished from true SNPs. To confront these problems, the rainbow trout transcriptome was sequenced from a doubled-haploid clonal line, a single fish with two completely homozygous and identical copies of each chromosome. This doubled-haploid clonal line was produced using androgenesis and gynogenesis techniques (Figure 11.5; Young et al., 1996; Robison et al., 1999). The assembled pyrosequencing data of the doubled-haploid transcriptome now serve as a reference sequence for functional genomics studies, including help in assembly and annotation of current genome sequence efforts, digital gene expression using RNA-Seq, and identification of pseudo SNPs allelic variants from paralogous sequences.

Application in SNP-based Genotyping

Whole-genome selection tools are being created to facilitate marker-assisted selection (MAS) and genetic improvement in plants and livestock. Genetic maps have been used for a wide range of species, including fish, to target

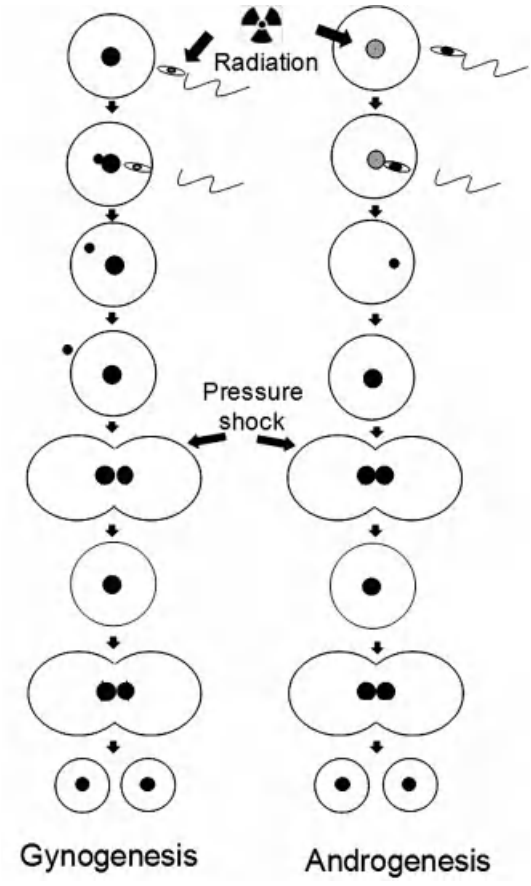

Figure 11.5 Rainbow trout are partially tetraploids causing identification of false SNPs and difficulties in assembly and annotation of shotgun sequences. To solve these problems, the rainbow trout genome and transcriptome are being sequenced from a doubled-haploid clonal line; a single fish with two completely homozygous and identical copies of each chromosome produced using androgenesis and gynogenesis.

discovery of allelic variation affecting traits with ultimate goal of identifying DNA sequences underlying phenotypes (Rexroad et al., 2008). Markers used in genetic maps have been identified by a range of molecular techniques such as RFLPs, RAPDs, AFLP, microsatellites, etc. To date, the linkage-based studies using these markers have not been successful in identifying genetic inheritance of complex traits for various reasons, including the cost of developing these markers. These markers are cumbersome to use in high-throughput genotyping protocols, and more importantly, they exist in low density and are not widely and evenly distributed in the genome. Consequently, these markers are not associated with unique sequences and are specific to the mapping population.

SNPs are abundant and genome-widely distributed. It is estimated that 90% of the genotypic variation in human arises from SNPs; 4–5 SNPs for every 1000 base pairs translate to 10,000,000 points of variation (Brookes, 2007). SNPs are codominantly inherited and easily adaptable to high-throughput automated genotyping. Therefore, they are most suitable for genome-wide association studies (Wang et al., 2008). Recent development of genome-wide SNP platforms has revolutionized genotyping technology by allowing a large number of SNPs to be genotyped in a large number of individuals. Currently, high-density mapping efforts for humans, model organisms, and agriculturally important species employ SNP markers.

The SNP-based genotyping technology enabled several genome-wide association studies to be carried out for the first time in complex human diseases, including diabetes (Lyssenko and Groop, 2009), asthma (Willis-Owen et al., 2009), and inflammatory diseases (Heap and van Heel, 2009). Genome-wide association technology provides a comprehensive and fair strategy to identify genes that control inheritance of complex animal production traits. Genome-wide association is rapidly replacing the classical candidate-gene studies and the traditional marker-based linkage mapping approaches that have dominated the gene discovery efforts for decades. Recently, whole-genome association studies in dairy and beef cows have identified QTLs associated with many economic traits, including growth, reproduction, immunity, milk yield, disease susceptibility, herd life, interval of calving to first service, age at first service, and temperament-related traits (Daetwyler et al., 2008; Druet et al., 2008; Gutierrez-Gil et al., 2008;

Kolbehdari et al., 2008; Maltecca et al., 2009; Matukumalli et al., 2009; Kirkpatrick et al., 2010; Snelling et al., 2010). A genetic variation map for chicken was constructed with 2.8 million SNPs and used to study feed conversion rate in broilers in a genome-wide-assisted selection context (Wong et al., 2004; González-Recio et al., 2009). Large-scale SNP genotyping revealed different demographic history in egg-laying chickens (Qanbari et al., 2010). Recently, the first genome-wide set of SNPs has been identified for sheep and was used to examine levels of genetic variability in a diverse sample of ovine populations based on geographic origin and known breed history (Kijas et al., 2009). A new SNP platform for swine has been released (Rothschild et al., 2007; Onteru et al., 2008; Illumina).

To date, scarcity of available complete genome sequences hindered genome-wide SNP marker development and usage for any aquaculture species. Until the genome sequences are available, preliminary studies have used EST-derived SNP markers for low-density linkage mapping in catfish, salmon, and cod (He et al., 2003; Smith et al., 2005; Hayes et al., 2007; Moen et al., 2008; Wang et al., 2008, 2010; Hubert et al., 2010). A rainbow trout genome sequence draft is underway. The rainbow trout genome project when available permits identification of SNP locations as the SNP-harboring sequences identified are anchored to the rainbow trout reference sequence. An important objective of the rainbow trout genome community is to create an affordable, high-throughput, genotyping platform using NGS technologies. The aforementioned rainbow trout genome consortium will develop a high-density SNP array (25–60K) for whole genome genotyping in rainbow trout. The SNP genotyping array will be produced using Illumina Infinium BeadArray. This genotyping platform can be used to predict genomic breeding values for fish production traits. Application of genome-wide selection for fish production traits including muscle growth and quality, feed efficiency, stress tolerance, and disease resistance will help in selecting improved germplasm for aquaculture. Outcomes of this project will facilitate development of similar platforms for other closely related aquaculture species such as Atlantic and Pacific salmon and char species.

Successful development of a high-density SNP array for whole genome genotyping depends on availability of large number of high-quality SNPs from rainbow trout. Previous efforts of SNP discovery from rainbow trout ESTs were unsuccessful. Technology of the Reduced Representation Libraries has been successfully used for SNP discovery in rainbow trout (Sanchez et al., 2009). The pyrosequencing technology has been used to identify SNPs from a reduced representation library constructed with genomic DNA pooled from 96 unrelated rainbow trout. The library represents broodstock population used in the breeding program of the USDA-NCCCWA. The reduced representation library was sequenced from 440 bp genomic fragments digested with the *Hae* III restriction enzyme and yielded 2 million reads (Figure 11.6). Newbler and Velvet algorithms were used to assemble the reads and create a reference sequence. Three different approaches were used in read assembly to screen for known repetitive elements. The ssaha2 algorithm was used to map the individual reads back onto the reference sequence and identified three sets of SNPs. A total of 22,022–47,128 putative SNPs on 13,140–24,627 contigs were identified. A sample of 384 putative SNPs was randomly genotyped on the 96 individual fish, initially used for library construction, to measure validation rate of putative SNPs, distinguish pseudo SNPs from paralogous loci because of the trout tetraploid genome, and examine the SNPs Mendelian segregation. A total of 183 (48%) of the putative SNPs were validated; of them, 167 markers were successfully incorporated into the rainbow trout linkage map. This study proves that the reduced representation libraries approach combined with pyrosequencing technology is an effective strategy for high-throughput

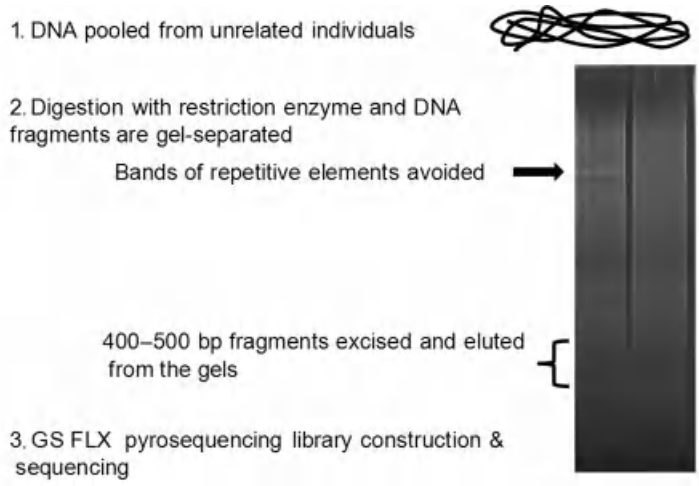

Figure 11.6 Overview of the Reduced Representation Libraries strategy used for SNP discovery. DNA pooled from unrelated individuals and digested with restriction enzyme. DNA fragment are excised (bands of repetitive elements are avoided) and sequenced. In rainbow trout, DNA was digested with HaeIII, bands of repetitive elements were not observed, libraries were sequenced using long-read GS FLX pyrosequencing.

discovery of a large number of putative SNPs in aquaculture species.

The rainbow trout SNP consortium currently use the RAD (restricted site associated DNA) approach for SNP discovery and genotyping by sequencing (Miller et al., 2007; Baird et al., 2008). SNP discovery using RAD sequencing of many double haploid clonal lines (Young et al., 1996) and samples from several out-bred populations will shortly be completed to produce a high-density genetic map for rainbow trout.

RNA-Seq Identified Genetic Markers for Fast/Slow Growth

Fast growth and nutrient efficiency of the aquaculture species are important desired traits essential in meeting rising food needs in the world. The increasing feed prices account for the largest percentage of production costs and affect sustainability and profitability of aquaculture production. Traditional phenotype-based selections have been used for decades and led to substantial improvements in growth rates and feed efficiency in some aquaculture species including rainbow trout (Bellmann et al., 2003; Quinton et al., 2007a, 2007b; Silverstein et al., 2009). MAS can be used in genetic programs to increase the genetic gain more rapidly than the conventional phenotype-based selection (Dekkers and Hospital, 2002; Andersson and Georges, 2004). To accelerate genetic improvement in plants and livestock, recent research efforts have been directed toward using markers that are tightly linked to target genes or QTLs as an alternative for phenotype-based selection. Some examples of successful applications of MAS include direct selection on single genes and utilization to maximize genetic gain for QTLs affecting economic traits in livestock breeding (Dekkers and Hospital, 2002; Dekkers, 2004; Villanueva et al., 2004; Pang et al., 2010). Several genetic markers are available and some have been used in commercial breeding applications in dairy and beef cattle, poultry, pigs, and sheep (Dekkers, 2004). A substantial number of growth and feed intake-associated markers were reported, including

mutations of the porcine melanocortin-4 receptor gene and the bovine vascular endothelial growth factor gene (Kim et al., 2000; Li et al., 2010a, 2010b). Applications of MAS in aquaculture selection have been delayed because of the lack of suitable markers and breeding programs. As explained earlier, SNPs are responsible for the greatest percentage of the individuals' genetic variations and are the most suitable markers for MAS, especially in genomic selection. Sequence polymorphism including SNPs located within or close to coding sequences can abort or alter the biological function of a protein, consequently, are particularly important in genetic selection.

A Whole Transcriptome Shotgun Sequence, RAN-Seq, approach has been used to detect SNPs from rainbow trout fish selected for fast growth versus slow-growing rainbow trout families (Salem et al., 2011). Pooled RNA samples (10 fish from 1 full-sib family each) were sequenced using short-read Illumina platform. Reads (average of 7.3 million each) were mapped to a reference transcriptome (161,818 transcript sequences). The reference transcriptome was sequenced and assembled from a double-haploid individual to minimize false SNPs because of paralogous loci (Salem et al., 2010). More than 300 putative type-I SNP markers with different frequencies in fast versus slow fish were identified. The ratio between the allele ratio of the fast sample and that of the slow sample (allelic imbalances) were considered at score >5.0 as an amplification and <0.2 as loss of heterozygosity. Figure 11.7 summarizes the RAN-Seq workflow and an example of a SNP identified in the fast growing fish.

Genotyping individual of the fast versus slow-growing fish, using the Sequenom iPLEX Genotyping platform, validated association of about 100 markers with the fast growth ($p < 0.01$). Markers were further evaluated on 354 individuals of a parent/offspring panel from the broodstock selection population at the USDA-NCCCWA. These fish were collected from ten full-sib families selected for fast growth, ten for slow growth in addition to three families of a control group. More than 50 markers showed strong association with growth rate. These markers are currently being incorporated into the rainbow trout linkage map. Many of these markers were mapped to genes of the mitochondrial aerobic respiration enzyme pathway. In addition to the RAN-Seq power in identification of SNPs, it revealed coordinated expression of growth-associated metabolic pathways in fast compared with slow fish. This study shows that Whole Transcriptome Shotgun Sequencing is a fast and effective means of detecting genetic polymorphism appropriate for marker development and genetic selection in aquaculture species without prior genome sequence or annotation.

Conclusion and Future Perspectives

Genome-wide MAS has potentials to improve aquaculture stocks, and molecular technologies are needed to enhance selective breeding. Recently, some molecular markers associated with aquaculture production traits have been identified (Reid et al., 2005; Perry et al., 2001). Nevertheless, there has been limited use of molecular genetics-based technologies to improve aquaculture production. SNPs are highly abundant, widespread, and evenly distributed markers that can be easily genotyped using high-throughput assays. SNPs have replaced traditional markers like microsatellite in most genomic analyses such as QTL mapping, linkage disequilibrium studies, and whole-genome selection. SNP-based whole-genome selection approaches have been developed to facilitate genetic improvement in livestock breeding programs (Daetwyler et al., 2008; Kolbehdari et al., 2008).

An SNP consortium has been organized to create a rainbow trout high-throughput SNP platform. SNPs have been identified using de novo deep sequencing of the transcriptome and reduced representation and RAD libraries of the genome. A large number of SNPs will be

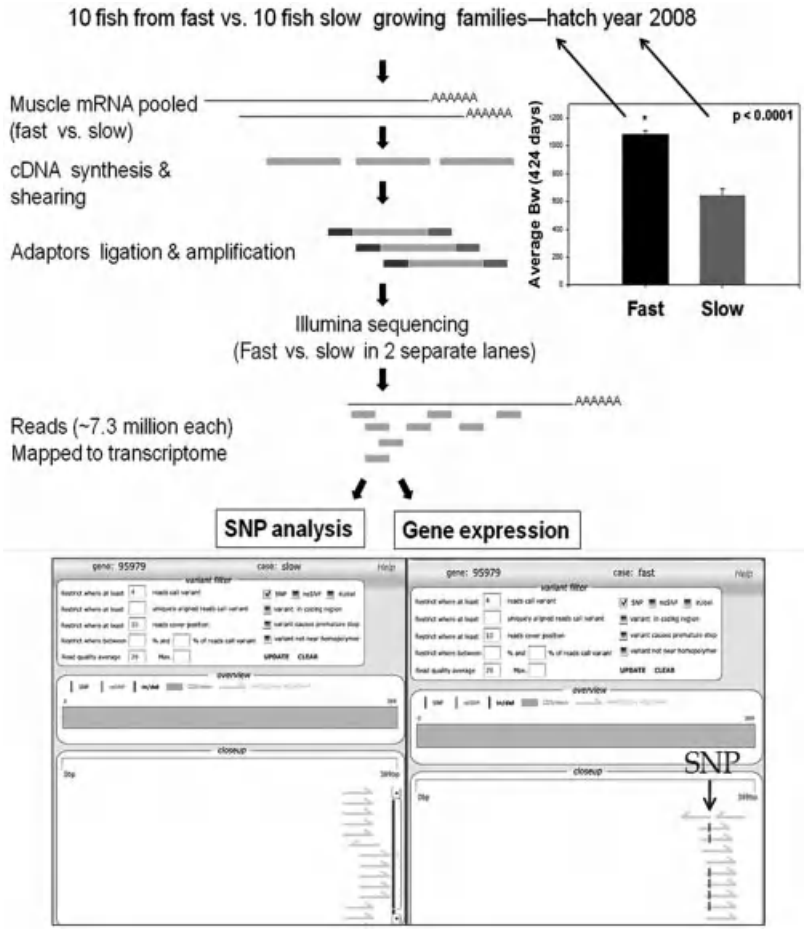

Figure 11.7 Upper panel, workflow of Whole Transcriptome Shotgun Sequencing (RNA-Seq) used to detecting genetic polymorphism in rainbow trout. Lower panel, an example of a type-I SNP marker identified in fast-growing (right) but not in slow-growing (left) rainbow trout fish population. See color insert.

validated and informative SNPs will be selected to construct an affordable (cents per marker genotype) 30–50K SNP assay for genotyping using the Illumina Infinium BeadArray. In addition, SNP markers will be mapped using fish populations from different regions, allowing development of important genomic tools for rainbow trout such as high-resolution, easy-to-use linkage maps, comparative maps, and integration of linkage and physical maps. This high-density SNP chip will be used to explore potential application of genomic selection in rainbow trout. Specifically, we could test accuracy of breeding values estimated from the SNP markers relative to phenotype-based estimated breeding values. The SNP platform can be used to facilitate genome-wide QTL analysis of important production traits like feed conversion efficiency and environmental tolerance. Identification of haplotypes associated with superior fish production traits will contribute to the sustainability and profitability of the aquaculture industry. In addition, the SNP technology will allow study of gene function through hypothesis-driven studies for aquaculture and biomedical and evolution research.

Alleles noted to be associated with phenotypes may be subjected to further gene function follow-up studies.

A minimum tiling path BAC clones were selected from a physical map. Sequencing and assembly of these clones will provide a complete draft of rainbow trout genome (Miller et al., 2011). The rainbow trout genome sequence permits identification of SNPs locations and selection of genome-wide equally distributed SNPs. In addition, comparative mapping of the rainbow trout genome to genomes of Atlantic salmon, catfish, zebra fish, puffer fish, and stickleback will provide valuable information about fish genome evolution, especially genome-scale gene duplications (auto-tetraploidization) in salmonids. Integration of linkage and physical maps depends on common set of markers or sequences on the physical map and the linkage map. The SNPs associated with BAC-ends, or gene-associate SNPs whose location is known on the physical map, will allow integration of the genetic linkage and physical maps. In addition, an important recent product of the rainbow trout genome project is a well-assembled and annotated reference transcriptome draft of the rainbow trout.

Finally, third-generation, single-molecule sequencing technology is now available with remarkable large-scale increase in read length, fast time, more informative data, and reduction in sequencing costs. The new technology holds even greater promises than the second-generation sequencing platforms for future developments of functional genomics tools of aquaculture species.

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Chapter 12
Functional Genomics Research of Atlantic Cod *Gadus morhua*

Matthew L. Rise

Abstract: Atlantic cod (*Gadus morhua*) aquaculture is a developing industry that may complement the wild cod fishery. Large-scale Atlantic cod genomics projects have generated resources such as cDNA libraries, sequence databases, microarrays, and single nucleotide polymorphism genotyping platforms. These resources enable genomics research on the genes and molecular pathways involved in aquaculture-relevant traits such as growth rate, robustness of defense responses, and responses to alternative diets. To date, Atlantic cod functional genomics research has focused largely on development, growth, and responses to immune stimuli and environmental stressors. In addition to providing valuable information on the genetic basis of biological processes of importance to aquaculture, functional genomics research will likely lead to the development of new tools and strategies for improving the health and performance of farmed cod.

Introduction

The Atlantic cod (*Gadus morhua*) fishery has historically been of cultural and economic importance in various parts of the world, including Atlantic Canada, Iceland, and Norway (Booman et al., 2011). Declines in wild Atlantic cod populations in Canadian waters led to a moratorium on cod fishing in the early 1990s, with devastating socioeconomic effects on coastal, rural communities in the region. The status of Atlantic cod stocks varies; some stocks have collapsed (e.g., Newfoundland cod of the Grand Banks), while others range from seriously overexploited (e.g., North Sea and Baltic cod) to satisfactory or moderately good (e.g., northeast Arctic cod and Icelandic cod; Marteinsdottir et al., 2005; Rise et al., 2009a). Unpredictable and variable harvests of wild Atlantic cod have contributed to the initiation of commercial farming of this species (Booman et al., 2011 and references therein). Atlantic cod aquaculture is an important alternative or complement to the wild cod fishery, with the potential to alleviate depletion of wild stocks (allowing them to recover) while still satisfying consumer demands (Booman et al., 2011).

Impediments to commercially viable Atlantic cod aquaculture include slow growth, early maturation, expensive and unsustainable feeds (e.g., based on fish oil and fish meal), sensitivity to handling stress or environmental stressors such as high water temperature, and infectious diseases (e.g., caused by viral, bacterial, and fungal pathogens such as nodavirus, *Aeromonas salmonicida* strains, and *Loma* species, respectively; Bowman et al., 2011 and references therein; Booman et al., 2011 and references therein). Aquaculture

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

genomics research projects may involve the construction and application of numerous tools such as libraries (e.g., cDNA or bacterial artificial chromosome (BAC)), expressed sequence tag (EST) databases, single nucleotide polymorphism (SNP) genotyping platforms, genetic and physical maps, DNA microarrays, and reference genomes. These genomics tools may be used to improve our understanding of the genetic basis of economically relevant traits, potentially leading to the development of markers for selecting superior aquaculture broodstock (Rise et al., 2007, 2009a, 2009b). In this chapter, I present progress to date in the area of Atlantic cod functional genomics research, which has focused on the identification and study of genes and molecular pathways involved in cod development, growth, responses to different diets, and responses to pathogens or environmental stressors (e.g., Lilleeng et al., 2007; Kortner et al., 2008; Rise et al., 2008, 2010; Feng et al., 2009; Hori et al., 2010; Johansen et al., 2011; Bowman et al., 2011; Booman et al., 2011; Drivenes et al., 2012). It is anticipated that an improved level of understanding of the genetic basis of these biological processes will lead to new strategies for improving Atlantic cod farming (e.g., through the development and use of optimized diets and genetic markers for selecting rapidly growing, stress-resistant, disease-resistant cod broodstock).

Aquaculture-related functional genomics research may include targeted gene discovery (e.g., using suppression subtractive hybridization (SSH) cDNA libraries enriched for genes involved in a biological process of interest), EST database (dbEST) creation and mining, DNA microarray development and application, quantitative reverse transcription—polymerase chain reaction (QPCR) assay development and application, and very recently, next-generation sequencing (NGS)-based transcriptomics (i.e., RNAseq). Each of these techniques has been employed in functional genomics research on Atlantic cod, as discussed in this chapter.

Large-scale Atlantic Cod Genomics projects have been conducted in Canada (the Atlantic Cod Genomics and Broodstock Development Project (CGP); www.codgene.ca) and Norway (The Cod Genome Project, http://codgenome.no). The Canadian CGP, supported in part by Genome Canada, Genome Atlantic, and the Atlantic Canada Opportunities Agency, built genomics tools such as normalized and SSH cDNA libraries, an dbEST containing over 150,000 ESTs, a 20,000 gene (20K) oligonucleotide microarray based on this dbEST (Table 12.1), numerous QPCR assays for defense-relevant transcripts, SNP resources, and a genetic linkage map (Rise et al., 2008, 2009a, 2010; Borza et al., 2009, 2010a, 2010b; Feng et al., 2009; Hori et al., 2010; Hubert et al., 2010; Booman et al., 2011; Bowman et al., 2011). The CGP resources are publicly available; the majority of the 229,094 Atlantic cod ESTs currently in the GenBank dbEST were submitted by the CGP. In addition, the CGP is the largest contributor of the 224,629 ESTs in Release 1.0 of the Dana-Farber Cancer Institute Atlantic cod Gene Index

Table 12.1 Atlantic cod (*Gadus morhua*) DNA microarray platforms.

Number of probes	Type of platform	Reference
Not provided	Spotted cDNA	Kortner et al., 2008
746	Spotted cDNA	Lie et al., 2009a
~16,000 (16K)[a]	Spotted cDNA	Edvardsen et al., 2011
20,000 (20K)	Spotted oligonucleotide	Booman et al., 2011

[a]This 16K platform (with ~ 3K probes from an embryo library) represents 7K genes (Drivenes et al., 2012).

(http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=atlantic_cod).

Norwegian cod genomics efforts have produced resources such as cDNA libraries, over 44,000 transcript sequences submitted to GenBank, a 16K cDNA microarray (Table 12.1), SNP resources, a genetic linkage map, NGS reads (e.g., for microRNA discovery, transcriptome characterization, and genome sequencing), BAC end sequences, and a genome assembly (Johansen et al., 2009, 2011; Moen et al., 2008, 2009; Edvardsen et al., 2011; Star et al., 2011). The Norwegian Cod Genome Project resources (e.g., NGS reads, transcriptome assembly, genome assembly, BAC end sequences) are publicly available through the Cod Genome Project Web site (http://codgenome.no).

In addition to the large-scale Atlantic cod genomics projects conducted in Canada and Norway, smaller studies have also contributed to Atlantic cod gene discovery, cDNA and gene characterization, QPCR assay development, and QPCR-based transcript expression analyses. Examples of these studies include the construction and sequencing (192 clones) of an SSH library for discovery of cod intestine transcripts that respond to different diets (Lilleeng et al., 2007), and cDNA and/or genomic characterization and QPCR-based mRNA expression analyses of various Atlantic cod genes including neuropeptide Y and cocaine and amphetamine regulated transcript (Kehoe and Volkoff 2007), interleukin genes IL-1β, IL-8, and IL-10 (Seppola et al., 2008), interferon γ (Furnes et al., 2009b), ghrelin and gastrin-releasing peptide (Xu and Volkoff, 2009), interferon-stimulated gene 15 (ISG15) (Seppola et al., 2007b; Furnes et al., 2009a), interleukin-22 (Corripio-Miyar et al., 2009), nonspecific cytotoxic cell receptor protein (Seppola et al., 2007a), hepcidin (Solstad et al., 2008), goose-type lysozyme (Larsen et al., 2009), four members of an anti-apoptotic Bcl-2-like gene family (Feng and Rise, 2010), activating transcription factor 3 (Feng and Rise, 2011), and Gaduscidin-1 and Gaduscidin-2, paralogous antimicrobial peptide-like transcripts (Browne et al., 2011).

In the remainder of this chapter, further details are provided on selected Atlantic cod functional genomics studies that have been conducted to date.

Functional Genomics Research on Atlantic Cod Defense Responses

Functional genomics research conducted by the CGP focused on the identification and study of genes that influence economically important traits for cod aquaculture. For example, Atlantic cod SSH libraries were constructed and sequenced for the targeted discovery of spleen and brain transcripts that respond to virus or viral mimic (Rise et al., 2008, 2010), spleen and head kidney transcripts that respond to bacterial antigens (Feng et al., 2009), and liver, skeletal muscle, and head kidney transcripts that respond to heat stress (Hori et al., 2010). The construction and application of SSH libraries have been explained elsewhere (e.g., Rise et al., 2007). Briefly, SSH libraries are enriched for genes upregulated in a "tester" sample relative to a "driver" sample (Diatchenko et al., 1996). Reciprocal SSH libraries may be created to identify genes that are upregulated or downregulated by a stressor such as a pathogen (Figure 12.1) or heat stress. Examples of the CGP's effective use of SSH libraries for targeted discovery of cod defense-relevant genes are provided later in this chapter.

In addition to SSH library construction and characterization, CGP functional genomics research also included QPCR assay development, dbEST creation and mining, and DNA microarray construction and hybridization. The first CGP functional genomics publications, focusing on SSH libraries and QPCR assays of SSH-identified transcripts, have dramatically improved the characterization of Atlantic cod defensome (i.e., all genes involved in defense responses; Rise et al., 2008, 2010; Feng et al., 2009; Feng and Rise, 2010, 2011; Hori et al., 2010). Additional information on the use of

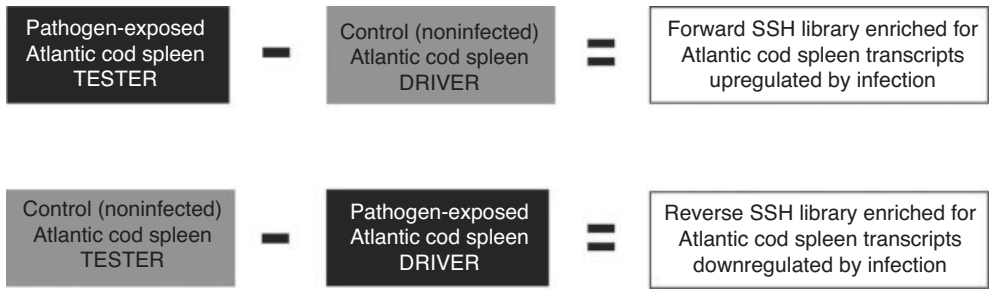

Figure 12.1 Examples of reciprocal SSH libraries designed for Atlantic cod immune gene discovery.

SSH libraries for targeted Atlantic cod defense-relevant gene discovery is provided later in this chapter.

As previously mentioned, the CGP developed an Atlantic cod dbEST (www.codgene.ca) containing over 150,000 ESTs (Bowman et al., 2011). In addition to approximately 20,000 ESTs from a total of 19 SSH libraries, the CGP EST data includes over 138,000 ESTs from a total of 23 normalized cDNA libraries representing various developmental stages, adult tissues, and conditions (e.g., heat stressed or immune stimulated; Bowman et al., 2011). The CGP ESTs were used to generate a resource of gene-associated SNPs, and 924 of these SNPs were included in a preliminary linkage map (Hubert et al., 2010). These resources will be valuable for quantitative trait locus studies and for identifying markers for selecting superior cod broodstock with economically important phenotypes such as rapid growth and resistance to infectious diseases (Hubert et al., 2010).

The CGP dbEST was used to design probes for inclusion in a 20,000-gene (20K) Atlantic cod oligonucleotide microarray that was recently built, quality tested, and validated using QPCR (Booman et al., 2011). The CGP 20K microarray platform (Table 12.1) is an important new tool for cod functional genomics research. Ongoing collaborative studies utilizing the Atlantic cod 20K microarray include (1) studies of egg, embryonic, and larval transcriptomes; (2) studies of global transcript expression responses to various diets; and (3) studies of global transcript expression responses to immune stimuli (e.g., pathogens or pathogen mimics), environmental stress (e.g., heat stress), and combinations of stressors (e.g., elevated ambient temperature and immune stimuli).

Atlantic Cod Anti-Viral Responses

Viruses that are members of the genus *Betanodavirus*, family *Nodaviridae*, include fish pathogens that threaten the global aquaculture industry (Rise et al., 2010; reviewed in Lang et al., 2009). Nodaviruses cause viral nervous necrosis, a disease reported in several species of marine finfish including Atlantic cod and can also reside in the central nervous systems of asymptomatic carriers (Rise et al., 2010 and references therein). Prior to CGP functional genomics research, little was known about Atlantic cod transcriptomic responses to viruses. A complete understanding of the genes and molecular pathways involved in Atlantic cod responses to pathogens is essential to the development of effective strategies (e.g., vaccines, and methods for marker-assisted selection of disease resistant broodstock) for combating emerging infectious diseases in farmed cod, and new tools (e.g., QPCR assays for molecular biomarkers of host immune responses) for studying the impact of pathogens on wild and farmed cod populations.

As a part of the CGP functional genomics research on cod immune responses, reciprocal SSH cDNA libraries were constructed and characterized to identify Atlantic cod spleen and brain transcripts that respond to asymptomatic high nodavirus carrier state and/or intraperitoneal (IP) injection with a synthetic double-stranded RNA viral mimic poly(I:C) (Rise et al., 2008, 2010). Rise et al. (2008, 2010) sequenced 3874 spleen and 3918 brain SSH library clones, and they functionally annotated the assembled sequences. Furthermore, they studied the expression of 13 SSH-identified transcripts using QPCR with individual spleen RNA samples (Rise et al., 2008) and 27 SSH-identified transcripts using QPCR with individual brain RNA samples (Rise et al., 2010) to assess biological variability. Rise et al. (2008) was the first publication on large-scale immune gene discovery in Atlantic cod, and the first to show that this species has a RIG-I RNA helicase viral recognition pathway. Rise et al. (2008, 2010) showed that both the cod spleen and brain mounted rapid and potent interferon pathway responses to IP injection of poly(I:C). Several poly(I:C)-responsive transcripts (e.g. ISG15, DHX58, and RSAD2) were strongly upregulated in the brains, but not the spleens, of asymptomatic high nodavirus carrier fish (Rise et al., 2008, 2010). CGP SSH libraries, ESTs, and QPCR assays represent important new genomics resources for researchers working on the immunology of Atlantic cod. In addition, the functional annotations of SSH-identified, QPCR-validated genes in Rise et al. (2010) provide valuable insight into the molecular pathways that respond in the brains of asymptomatic high nodavirus carriers (e.g., apoptosis-relevant genes BNIP2 and NUPR1 were significantly downregulated in high virus brains) and will lead to new hypotheses regarding the nature of the interactions between the vertebrate brain and viruses that infect the central nervous system. Many genes identified in these SSH libraries were included in the design of the CGP 20K microarray (Booman et al., 2011), making it a valuable new tool for functional genomics research on Atlantic cod responses to viruses.

Atlantic Cod Anti-Bacterial Responses

A. salmonicida subspecies *salmonicida* are Gram-negative bacteria that cause typical furunculosis, a bacterial septicaemia of coldwater fish (Feng et al., 2009). In addition to this subspecies, there are other *A. salmonicida* subspecies that produce atypical forms of furunculosis (Feng et al., 2009). Both typical and atypical *A. salmonicida* can cause disease in farmed Atlantic cod (Samuelsen et al., 2006; Feng et al., 2009). In order to identify genes that are involved in the Atlantic cod immune response to bacterial antigens, Feng et al. (2009) constructed reciprocal SSH libraries enriched for transcripts that were differentially expressed in the spleen and hematopoietic kidney (i.e., head kidney) of juvenile Atlantic cod following IP injection with formalin-killed, atypical *A. salmonicida*. From these SSH libraries, Feng et al. (2009) obtained 4154 ESTs that were assembled and functionally annotated. This CGP functional genomics study revealed that bacterial antigen stimulation caused changes in many biological processes, including chemotaxis, regulation of apoptosis, antimicrobial peptide production, and iron homeostasis (Feng et al., 2009). SSH-identified transcripts shown by QPCR to be significantly upregulated by *A. salmonicida* included transcripts encoding interferon regulatory factor 1 (IRF1), a small inducible cytokine, and interleukin 8 (Feng et al., 2009). This study was the first large-scale targeted discovery of bacteria-responsive genes in Atlantic cod, and the first to demonstrate upregulation of IRF1 transcript in fish immune tissues following stimulation with bacterial antigens (Feng et al., 2009). Many genes identified in these SSH libraries were included in the design of the CGP 20K cod microarray, contributing to the utility of this new microarray platform for functional genomics studies of Atlantic cod

responses to bacterial pathogens (Booman et al., 2011). Functional genomics research on cod immune responses may facilitate the development of management practices, markers and methods for selecting disease-resistant broodstock, and new vaccines and therapeutics to combat disease outbreaks in Atlantic cod aquaculture (Feng et al., 2009).

Atlantic Cod Response to Heat Shock

Temperatures can rise rapidly (e.g., increase of 8°C in less than 12 hours during thermocline inversions) in Atlantic cod aquaculture cage sites and can approach upper critical temperatures (i.e., seawater temperatures that are lethal) (Hori et al., 2010). Cod in sea cages cannot completely avoid these increased temperatures, and therefore may be exposed to heat stress (Gollock et al., 2006; Hori et al., 2010). Elevated temperatures can influence expression of fish immune-relevant transcripts and proteins and increase susceptibility to infectious disease (Varsamos et al., 2006; Pérez-Casanova et al., 2008). The mechanisms by which heat stress may alter fish immune responses to pathogens remain poorly understood. As part of the CGP functional genomics research efforts, Hori et al. (2010) built and sequenced SSH libraries to identify heat-stress-responsive Atlantic cod transcripts. They generated a total of 5980 ESTs from liver, head kidney, and skeletal muscle SSH libraries, and the assembled sequences were functionally annotated (Hori et al., 2010). Protein folding was among the highly represented gene ontology terms in these libraries. Using QPCR for SSH-identified transcripts, Hori et al. (2010) demonstrated that several chaperone-encoding transcripts (HSP90a, HSP70-1, HSP47, GRP78, and GRP94) were significantly upregulated in all three tissues, and one immune-relevant transcript (TLR22) was significantly downregulated in head kidney, following heat shock. Hori et al. (2010) was the first to use high-throughput genomic techniques to study the Atlantic cod response to heat stress, greatly improving our understanding of how heat stress may negatively impact the physiology and health of this economically important fish species. Many genes identified in these SSH libraries were included on the CGP 20K oligo microarray. The 20K cod microarray platform is currently being used to study the impact of elevated ambient temperature on immune tissue global transcript expression responses to a viral mimic poly(I:C) (manuscript in preparation).

Characterization and Expression Studies of Atlantic Cod Bcl-2-Like Genes

Members of the Bcl-2 family of genes and proteins are central regulators of apoptosis (Feng and Rise, 2010). Given the anti-apoptotic functions of Bcl-2 family proteins NR-13, Mcl-1, and Bcl-X in vertebrates, and the role of apoptotic regulation in immune responses, Feng and Rise (2010) characterized these genes in Atlantic cod and studied their constitutive and immune-responsive transcript expression. Feng and Rise (2010) identified four anti-apoptotic Bcl-2 family genes in Atlantic cod: NR-13, Mcl-1, Bcl-X1, and Bcl-X2. For cod NR-13, Mcl-1, and Bcl-X1, Feng and Rise (2010) used bidirectional rapid amplification of cDNA ends, genome walking, and other molecular techniques to sequence the full-length cDNA, resolve the gene structure, and obtain and analyze upstream promoter element-containing sequences. In addition, they obtained the partial cDNA sequence and partially resolved the gene structure of Atlantic cod Bcl-X2 (Feng and Rise, 2010). For all four cod Bcl-2 family transcripts, Feng and Rise (2010) used QPCR to examine constitutive transcript expression in six tissues as well as immune tissue transcript expression responses to bacterial antigens (formalin-killed, atypical *A. salmonicida*) or poly(I:C). They found that NR-13 and Bcl-X2 transcripts were most highly expressed in gill, while Mcl-1 and Bcl-X1 transcripts were

most highly expressed in blood (Feng and Rise, 2010). Furthermore, they found that NR-13, Mcl-1, and Bcl-X2 transcripts were significantly upregulated in spleen following IP injection of poly(I:C), whereas only NR-13 transcript was significantly upregulated in spleen after IP injection of bacterial antigens (Feng and Rise, 2010). This was the first report of fish Bcl-2-like transcript expression responses to bacterial and viral antigens. The molecular phylogenetic analysis and gene structure analysis in Feng and Rise (2010) provided important insights into the evolution of vertebrate Bcl-2-like genes.

Atlantic Cod DNA Microarray Platforms for Functional Genomics Research

Before large numbers of ESTs were available, several small-scale microarray platforms were developed. Kortner et al. (2008) generated 150 ESTs from an SSH library designed to be enriched for androgen-responsive cod ovary transcripts, and they built a cod gonadal cDNA microarray that included these SSH-identified transcripts. Transcripts identified in these SSH libraries, and some additional genes, were included in a spotted cDNA microarray (Table 12.1) that was used to further study cod oocyte responses to androgens (Kortner et al., 2008). The CodStress array, designed to include over 700 transcripts encoding proteins involved in stress and immune responses (Table 12.1), has been used for toxicogenomics studies (e.g., to identify cod liver transcripts that respond to environmental toxicants such as alkylphenols; Lie et al., 2009a, 2009b).

Two large-scale Atlantic cod DNA microarrays have been recently built and quality tested: a 16,000 gene (16K) cDNA microarray platform (Edvardsen et al., 2011) and the CGP 20K oligonucleotide (oligo) microarray platform (Booman et al., 2011). Transcripts selected for inclusion on the 16K cDNA microarray platform arose from a set of over 44,000 ESTs from cDNA libraries representing different tissues and developmental stages; the 16K microarray (representing 7000 genes) has been used to (1) compare the transcriptomes of four different tissues (pituitary gland, pylorus, spleen, and testis) from male cod (Edvardsen et al., 2011), (2) identify genes and molecular pathways that may be involved in distended gut syndrome of larvae (Kamisaka et al., 2010), and (3) study global transcript expression during embryonic and early larval development (Drivenes et al., 2012). The 50-mer oligonucleotide probes on the CGP 20K microarray platform were designed based on a set of over 150,000 ESTs from cDNA libraries representing different tissues, developmental stages, and conditions (e.g., heat-stressed or immune-stimulated; Booman et al., 2011; Bowman et al., 2011). This microarray was validated using QPCR and has been used to study the Atlantic cod spleen transcriptome response to formalin-killed, atypical *A. salmonicida* (Booman et al., 2011).

Conclusions and Future Perspectives

Atlantic cod is an important species for the global fisheries and aquaculture industries. Large-scale cod genomics projects, such as the CGP (Canada) and the Cod Genome Project (Norway), have developed valuable genomics tools, including DNA microarrays, sequence databases, sequence assemblies, and SNP genotyping platforms. Challenges facing the Atlantic cod aquaculture industry include early sexual maturation, slow growth, unsustainable diets, emerging infectious diseases, and susceptibility to environmental stressors such as heat stress. Functional genomics research can identify the key genes and molecular pathways involved in traits of interest to the aquaculture industry. With sequencing throughput increasing and cost decreasing, the opportunity is now available to use NGS to identify SNPs in exons, introns, and/or regulatory regions of all trait-relevant candidate genes (e.g., all growth-relevant genes, all immune-relevant genes, all

stress-responsive genes, etc.). Future research will likely focus on the validation of markers (e.g., SNPs) for production-relevant traits (e.g., delayed sexual maturation, rapid growth, resistance to disease, resistance to environmental stress, and favorable response to plant-based diets) that may be used in marker-assisted selection of superior Atlantic cod broodstock. As direct functional studies are more difficult and expensive, it is likely that cod functional genomics will be conducted through genome-scale expression analysis, identification of expression candidate genes, whole genome association studies using SNP markers and other types of markers with their associated performance and production traits, or whole genome linkage disequilibrium analysis of various aquaculturally important traits.

Acknowledgments

The functional genomics research outlined in this chapter was performed by several research groups. I would like to extend particular thanks to all of the scientists who contributed to the functional genomics components of the Atlantic Cod Genomics and Broodstock Development Project (CGP), funded in part by Genome Canada, Genome Atlantic, and the Atlantic Canada Opportunities Agency (ACOA). In addition, Atlantic cod functional genomics research in my laboratory has been supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant and a Canada Research Chair (Tier 2) in Marine Biotechnology.

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Chapter 13
Catfish Functional Genomics: Progress and Perspectives

Eric Peatman

Abstract: Catfish (*Ictalurus* sp.) is the predominant aquaculture species in the United States and an important research model for immunology, toxicology, and neurobiology. Functional genomics studies have been limited until recently in the species, however, by incomplete transcriptomic resources and tools. This chapter details the historical approaches taken to enable functional genomics studies in catfish and outlines early and ongoing research efforts using both microarrays and RNA-seq focused particularly on understanding disease responses. A consideration of potential and pitfalls of RNA-seq-based functional genomics research is also included.

Introduction

Aquaculture has enjoyed the fastest growth of any agricultural sector over the last three decades. As wild fisheries continue to decline and population demands for high-quality protein food sources rise, aquaculture is projected to continue to see explosive growth. While culture practices for some aquaculture species are now well established, the industry as a whole is still in its infancy when compared with traditional livestock species such as poultry, swine, and cattle. The tremendous gains in productivity seen in these species over the last 50 years have come from rigorous efforts focused on selecting the best performing individuals (genetics) to grow in the best environments. Initial selection efforts focused on gross phenotypes are now increasingly complemented or replaced by selection based on molecular genetic profiles including gene or genomic expression signatures and/or single nucleotide polymorphism (SNP) patterns associated with trait performance. Establishing these important connections between genetic information (DNA sequence) and the ultimate biological function forms the core of the study of functional genomics.

Functional genomics efforts in aquaculture species are relatively recent, hindered initially by the high cost of sequencing, the laborious nature of quantifying gene expression by Northern blot or RT-PCR, low levels of funding for aquaculture genomics research, and other factors. However, hard work over the last decade, coupled with the steady advance of genomic techniques and technologies, have opened up the field of functional genomics as a viable tool for selection in aquaculture. Here, we review advances in catfish (*Ictalurus* spp.) functional genomics to date and reflect on future directions for catfish functional genomics research.

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

Catfish Industry Overview

Ictalurid catfish (primarily *Ictalurus punctatus*, channel catfish; *I. furcatus*, blue catfish; and their hybrid) are the primary aquaculture species in the United States, accounting for 60% of all aquaculture production. Culture of catfish is centered in the rural Southeastern part of the United States, predominantly Mississippi, Alabama, and Arkansas. Long a regional favorite, ictalurid catfish now face growing competition from imported catfish, tilapia, and lower cost protein choices. Simultaneously, rising feed prices have greatly strained the profitability of catfish farming. As a result, less than 500 million pounds of catfish were processed in 2010, a reduction of close to 30% compared with the industry high in 2003. Acreage has been reduced from 196,750 acres in 2002 to 99,600 acres in 2011, amounting to almost a 50% reduction (Hanson and Sites, 2010). Many catfish farmers are struggling to carry on operations as it becomes clear that current production practices may not be viable in face of new economic realities. It is apparent that better production technology and genetically superior catfish are necessary components to ensure long-term sustainability and growth of the industry. Past and continuing efforts aimed at increasing industry efficiency and profitability include genetic and genomic research and selective breeding, establishment of best management practices, and improved, intensive raceway systems.

Although channel catfish (*I. punctatus*) is the major species used in catfish aquaculture, blue catfish (*I. furcatus*) possess several superior traits, including higher resistance against the gram-negative bacterium (*Edwardsiella ictaluri*) that causes enteric septicemia of catfish (ESC), higher resistance to proliferative gill disease, greater processing yield because of its uniform body shape, and better seinability because of its higher position in the water column. The channel catfish female × blue catfish male F1 hybrid exhibits a high level of heterosis and is a preferred genotype for aquaculture production, but because of reproduction isolation, artificial fertilization is required for the production. While significant progress has been made toward improving the efficiency of hybrid production, the quantities produced cannot currently meet demand (Liu, 2011a). Therefore, selection programs and genomics research in catfish must include work on both parental species with the ultimate goals of production of superior hybrid catfish and/or introgression of superior genes from blue catfish into channel catfish (Liu et al., 2003; Kucuktas et al., 2009).

Focus Areas for Catfish Functional Genomics Research

Several key production traits and their underlying biological processes are potential targets for investigation using functional genomics in catfish. These include growth rate, feed conversion efficiency, disease resistance, stress tolerance, processing yield, temperature tolerance, and tolerance of low dissolved oxygen. Catfish researchers are fortunate in that several geographically isolated strains of channel catfish and blue catfish are known to display differing phenotypes for the listed traits, providing natural material for connecting genotype and phenotype. Additionally, as mentioned earlier, channel catfish and blue catfish have notable differences in production performance.

Growth rate and feed conversion efficiency are particularly critical areas of research as catfish feed prices have risen precipitously and now account for close to 60% of variable costs. Faster growing fish mean a shortened growing cycle, reduced risk, and reduced overhead costs. Perhaps even more critical is the utilization of functional genomics to understand and select fish resistant to important catfish pathogens. These include *E. ictaluri*, columnaris (*Flavobacterium columnare*), and emerging, highly virulent strains of *Aeromonas hydrophila*. These diseases are capable of

causing devastating losses under certain conditions and add tremendous uncertainty to the catfish production process. Stress tolerance and low dissolved oxygen tolerance are important because these traits affect survival rates, disease susceptibility, feeding response, and energy consumption because of the use of emergency aeration. Stress tolerance is also important immediately before processing of catfish during transportation because stress of catfish can lead to degradation and discoloring of the catfish filet. Elucidation of genetic pathways controlling these traits in catfish, and identification of genetic variation that leads to improved performance, will permit correlation of levels of gene expression and diagnostic proteins with phenotypes (Liu et al., 2008).

Functional genomics can also be used to answer questions of interest in disciplines outside aquaculture. These include, for example, an understanding of sex determination mechanisms in fish, an understanding of the development of particular anatomical and physiological features (catfish barbels, lack of scales, electroreceptors), and research on novel immune response strategies in primitive teleost fish. In many cases, these investigations are enhanced by comparisons between catfish and appropriate control species (i.e., zebrafish, fugu, chicken). These comparative functional genomics are critical not only in our understanding of developmental processes but also in transferring knowledge of gene function from historical model species to aquaculture species (Elgar, 2004).

Historical Development of Catfish Functional Genomic Resources

While functional genomics can and should include an examination of the translated protein (proteomics) and metabolic products and pathways (metabolomics), slower technology development and, until recently, the relative paucity of DNA sequences have largely limited functional genomics research in aquaculture species to research on the transcriptome. The transcriptome is the mRNA pool representing the expressed set of transcripts within a given cell, tissue, or organ at a given point in time (Goetz and MacKenzie, 2008).

cDNA Library Construction and Expressed Sequence Tag Sequencing

Early on, efforts in catfish functional genomics focused on capturing the catfish transcriptome. Catfish cDNA libraries were generated from embryonic, juvenile, and adult tissues of catfish under varying physiological conditions such as tissues from pathogen-infected fish and cells exposed to lipopolysaccharide (LPS; Ju et al., 2000; Cao et al., 2001; Karsi et al., 2002; Kocabas et al., 2002a; Nonneman and Waldbieser 2005; Li and Waldbieser 2006; Li et al., 2007). A combined strategy of both normalization and subtraction coupled with negative-hybridization selection for novel clones (Li et al., 2007) has also been used to maximize the detection of rare transcripts. In total, 32 unidirectional plasmid cDNA libraries were generated (ten normalized libraries) in our laboratory, as detailed in Wang et al. (2010). Transcriptome efforts were directed at both channel catfish and blue catfish, as explained earlier. Sequencing of expressed sequence tags (ESTs) occurred both in-house and in collaboration with the Department of Energy's Joint Genome Institute. At the conclusion of the main EST sequencing efforts in 2010, close to 500,000 ESTs had been deposited in GenBank and represented 111,578 unique sequences (45,306 contigs and 66,272 singletons). Most importantly, EST development enabled the identification of 14,776 unique genes from ictalurid catfish. This gene set is available both in NCBI's GenBank as well as in an integrated form at the catfish genome database, cBARBEL (www.catfishgenome.org; Lu et al., 2011). Additionally, the catfish EST sequences provided a platform for the identification and characterization of full-length cDNA clones. A total

of 10,037 channel catfish and 7382 blue catfish putative full-length cDNA were identified from the catfish EST assemblies, with efforts ongoing to sequence complete full-length cDNA sets (Wang et al., 2010).

Next-Generation Sequencing for Transcriptome Discovery

The development of new platforms for ultra-high-throughput DNA sequencing (next-generation sequencing) has greatly reduced the cost, complexity, and time required to develop transcriptome resources for a species (Torres et al., 2008). This was well illustrated by a recent project utilizing Illumina Genome Analyzer IIx and HiSeq 2000 next-gen platforms. While ostensibly aimed at generation of gene-associated SNPs from ictalurid catfish, this project also demonstrated the power of next-generation sequencing to capture a large pool of gene transcripts. Sequencing of four Illumina lanes of mixed tissue RNA from channel catfish and blue catfish generated over 35 billion base pairs of sequences, including 220 million reads from channel catfish and 280 million reads from blue catfish. After assembly and BLAST, our analysis revealed over 5000 novel genes that had not been captured in previous EST efforts. Given a price tag around $5000 (US) and a timeframe of less than six months from tissue extraction to completion of data analysis, the results represented an astounding leap forward from our previous cDNA sequencing approaches. The next-gen results also contain a large number of unique unknown transcripts and rarely expressed noncoding RNA that will be valuable in genome annotation and gaining an understanding of the complete transcriptome in the future (Liu et al., 2011b). Ongoing projects in our laboratories are utilizing Illumina sequencing for further transcriptome development as well as high-throughput sequencing of full-length cDNAs, and a number of targeted gene expression profiling (RNA-seq) experiments (refer to the following text).

Utilization of Transcriptome Resources for Macro- and Microarray-based Studies

As initial sequencing in catfish provided sufficient information to design primers and clone genes, the expression of several candidate genes of known functional importance in model species were investigated in catfish. For example, Kim et al. (2000) examined skeletal alpha-actin, Kocabas et al. (2002b) characterized catfish myostatin, and Leonard et al. (2001) sequenced and reported expression patterns of neuropeptide Y sequences. Expression and characterization of a priori gene targets such as mannose-binding lectin (Zhang et al., 2012) continue to add to our understanding of gene and gene pathway functions in catfish. However, functional genomics is usually differentiated from these small studies by its focus on large-scale, high-throughput analysis of gene expression and its ability to identify novel, previously unconsidered gene functions.

Cold Acclimation Macroarray Studies

The first large-scale analysis of gene expression in catfish utilized a low-density macroarray of 660 channel catfish cDNAs spotted onto a nylon membrane. Expression of transcripts from the catfish brain in response to cold acclimation was assayed at four time points (0, 2, 24, and 48 hours) after a shift from 24°C to 12°C (Ju et al., 2002). A rapid induction of signal transduction and chaperone genes suggested that both de novo synthesis of cold-induced proteins and modification of existing proteins were required as part of the adaptive response to decreased ambient temperatures.

The same array was utilized to examine differentially expressed genes in a cold-adapted ictalurid *Ameiurus catus*, white catfish, after cooling (Kocabas et al., 2004). In contrast to channel catfish that reduce their feed

consumption markedly in winter months, white catfish continue to feed actively. Brain RNA from white catfish subjected to different temperatures was hybridized with the array. Seven genes (ependymin, ribosomal protein L41, ribosomal protein S27, 16S mitochondrial rRNA, and three unknowns) were upregulated when white catfish were cultured at low temperature. Twenty-four genes (11 known and 13 unknown) were downregulated in the brain of white catfish cultured under cold acclimation condition with an initial temperature of 24°C and a final temperature of 12°C. These genes include nonselenium glutathione phospholipid hydroperoxide peroxidase, creatine kinase, bithoraxoid-like protein, NADH dehydrogenase, translation elongation factor alpha, ribosomal protein L7a, and Von Hippel-Lindau-binding protein 1.

First-Generation In Situ Microarray Studies

As catfish EST resources became more plentiful, a pilot study was carried out to measure catfish gene expression on a larger scale (Li and Waldbieser, 2006). Two duplicated sets of ten 24-mer oligonucleotides (features) were designed using proprietary software (NimbleGen Systems Inc., Madison, WI) to represent each EST; one set of oligos was perfectly matched and positioned throughout the sequence, and the second set of duplicates contained two mismatched bases at positions #13 and #19 from the 5′ end. Oligonucleotides (24-mer) were synthesized on the microarray surface (NimbleGen) using in situ maskless array synthesis technology (Singh-Gasson et al., 1999; Nuwaysir et al., 2002). A total of 382,409 features were synthesized within the array area. Of these, 379,652 were catfish specific and the remaining 2757 were used for quality control of oligonucleotide synthesis and hybridization and for signal normalization. The resulting platform included 18,989 catfish ESTs, 38% of which (7182) could be putatively annotated as non-repetitive genes based on significant BLASTX similarity with sequences in GenBank.

The first-generation array described earlier was utilized to examine gene expression in the channel catfish spleen following intraperitoneal injection of LPS followed by tissue sampling at 2, 4, 8, or 24 hours postinjection. Biotinylated complementary RNA was produced by in vitro transcription of total RNA from spleen and used to probe the microarray. Following hybridization, the microarrays were scanned with an Axon GenePix 4000B scanner (Molecular Devices Corp., Union City, CA) at 5 mM resolution, and data were extracted from the raw images with NimbleScan software (NimbleGen, Madison, WI). A total of ten microarrays were used for this project: two replicates for the control pool and one microarray for each of the eight LPS-stimulated individuals (two from each time point). Relative signal intensity ($\log_2$ transformed) was generated for each feature using the robust multi-array average (RMA) algorithm (Irizarry et al., 2003). The signal intensity was background corrected based on the quantile normalization process (Bolstad et al., 2003; Bioconductor open source software for bioinformatics (http://www.bioconductor.org)) for all microarrays from the entire experiment (under uniform conditions) using data from only perfectly matched oligos. Experimental data and platform file was submitted to the NCBI Gene Expression Omnibus (GEO) under accession number *GSE3261*.

To analyze changes in gene expression following LPS injection, the normalized data were analyzed using Significance Analysis of Microarrays (SAM; Tusher et al., 2001) from the TIGR Multiexperiment Viewer analysis software package (TIGR Microarray software (http://www.tigr.org/software/microarray.shtml)) with a two-class unpaired design for each time point. The RMA value from perfectly matched oligos was used to calculate fold differences in expression (average signal intensity of treated animals divided by the average signal intensity of control animals). Cutoff values dictated that genes manifest changes of

at least twofold with an estimated global false discovery rate of <10%. Analysis of the data using SAM revealed 138 sequences that were differentially expressed after LPS treatment. Sixty-four genes were upregulated by LPS exposure, including cytokines and chemokines such as interleukin (IL)-1b, chemokine (C-C motif) ligand 4 (CCL4), a small CXC/IL 8-like chemokine, and chondromodulin II. Transcriptional factors such as NF-kb p100 subunit and NF-kb inhibitor α-like proteins A and B, interferon regulatory factor I, and adaptor-related protein complex 1 were also upregulated at least twofold. Expression of toll-like receptor 5 was upregulated at 2–4 hours postexposure in spleen. On the other hand, 74 genes were significantly downregulated in response to LPS, including immunoglobulin light and heavy chains, major histocompatibility complex (MHC) class II antigens, invariant chain-like protein 2, an NK lysin-like protein, complement C3, hemoglobin alpha, and a CXC chemokine receptor. Nine expressed sequences representing varying levels of expression were analyzed using RT-PCR to verify the microarray results. Linear regression of log-transformed expression data demonstrated a strong positive correlation between the two technologies (Li and Waldbieser, 2006; Liu et al., 2008).

The first-generation Nimblegen array studies were important in demonstrating the feasibility of the technology. However, the greatest limitation was the small number of genes that could be identified based on BLAST analysis. In total, only 55 genes could be annotated from all time points, making it difficult to carry out subsequent gene ontology or pathway analyses.

Second-Generation In Situ Microarray Studies

Additional sequencing of blue catfish ESTs and immune-relevant transcripts from both species allowed the subsequent expansion of the catfish EST resources and provided the impetus for the construction and utilization of a second-generation catfish microarray. Catfish EST sequences were re-clustered using the ContigExpress program of the Vector NTI software suite (Invitrogen, Carlsbad, CA) to ensure a unique gene set. Channel catfish and blue catfish repeat-masked sequences were annotated by BLASTX comparison against the NCBI nonredundant (nr) protein database (*E* value cutoff 10^{-5}). These sequences were used to produce a second-generation catfish microarray on the NimbleGen platform. Unlike spotted cDNA or oligo arrays, in situ arrays can be easily and inexpensively reconfigured by updating the feature database. For each EST represented on the microarray, at least 12 24-mer oligonucleotides were designed in the same way as for the 19K array. Six were perfect match oligos selected along the length of the sequence, while the other six were duplicates of the first but with two mismatched bases at the #6 and #12 positions from the 5′ end (Peatman et al., 2007, 2008). Reducing the level of sequence redundancy permitted synthesis of oligos corresponding to 28,518 genes on the array. As with the 19K microarray, sensitivity and reproducibility spike-in controls were included in the 28K oligo array platform (Liu et al., 2008).

The 28K array was utilized in two experiments aimed at capturing and comparing gene expression in the liver following artificial challenge with *E. ictaluri* (GEO accessions numbers *GSE6105* and *GSE6350*). As detailed earlier, blue catfish have markedly lower susceptibility to ESC when compared with channel catfish. Hepatic gene expression was examined 3 days postexposure (Peatman et al., 2007, 2008). In channel catfish, gene expression levels were compared with the levels observed in noninfected control catfish. After data normalization and calculation of RMA values, the resulting expression intensity values were analyzed using SAM. Identical criteria as in Li and Waldbieser (2006), a two fold or greater change in expression and a global false discovery rate of 10%, were applied to identify up- or downregulated genes. A total of 301 transcripts

were significantly upregulated, of which 207 were found to represent unique genes. Only six transcripts (five unique) were significantly downregulated. The redundant transcripts resulted either from blue and channel putative orthologues of the same gene or multiple transcripts from nonoverlapping regions of a large cDNA being included on the microarray. The data showed a wide range of transcriptional activity following *E. ictaluri* exposure. Fourteen genes were upregulated from 10- to 85-fold following infection; 16 genes were upregulated from five- to tenfold; 27 genes were upregulated from three- to fivefold; and 150 genes were upregulated from two- to threefold.

A conserved acute phase response was evident in channel catfish after infection. At least 35 of the 127 unique, BLAST-annotated transcripts represented acute phase proteins (APP; Bayne et al., 2001), including coagulation factors, proteinase inhibitors, transport proteins, and complement components. Many of the APPs were upregulated greater than fivefold, but several APP included on the microarray were not upregulated at the studied time point and these included mannose-binding lectin 2, serum amyloid P, and heparin cofactor II. Two subgroups of APP, iron transport–homeostasis proteins and complement components, were represented by particularly high numbers of upregulated transcripts. Transcripts representing at least 15 unique complement components or inhibitors were upregulated twofold or greater following infection. These included a short transcript likely representing C1q upregulated 15.3-fold increase; ficolin-like genes upregulated as much as 32-fold; complement C2/Bf; several C3 isoforms; complement components C4 and C5; complement components C7, C8, and C9 active in the membrane attack complex, and several complement regulatory proteins including MAC inhibitor CD59, C1 inhibitor, and Factor H. The most highly upregulated group of functionally related catfish genes was composed of genes involved in iron homeostasis. These included intelectin, the most highly upregulated gene observed at >85-fold, haptoglobin (>34-fold), haemopexin (>25-fold), ceruloplasmin (8.5-fold), transferrin (greater than sevenfold), and ferritin (greater than twofold). Trends in expression measured by the microarray were confirmed by quantitative RT-PCR analysis (Peatman et al., 2007).

Examination of gene expression changes in blue catfish liver at the same postexposure time point revealed that both species shared a wide spectrum of similarities in gene expression profiles. A total of 126 transcripts, believed to represent 98 unique genes, were upregulated in blue catfish following infection. While a smaller set than identified in channel catfish, a similar acute phase response and strong induction of complement components and iron regulatory genes were observed. However, significant differences were also observed between the expression profiles of the two species. A total of 58 genes were differentially expressed in blue catfish liver but not in channel catfish liver at day 3 after infection, including immune-relevant genes such as CC chemokine SCYA106, MHC class I alpha chain and matrix metalloproteinase 13 (MMP13). The microarray results were extended with additional quantitative RT-PCR analyses, which found, among other results, that MMP13 was 21.8-fold upregulated in channel catfish liver 24 hours postexposure and only 1.6-fold upregulated in blue catfish liver at this early time point in the controlled pathogen exposure (Peatman et al., 2008).

Microarray experiments are often described (and criticized) as gene-fishing expeditions, since the set of resulting genes with perturbed expression patterns is usually unknown until the data has been analyzed. We have found that the microarray experiments conducted to date in our laboratory have been useful in revealing candidate genes for follow-up study that would have escaped our notice using a purely a priori approach. Over the last several years, we have further characterized many of these genes through Q-RT-PCR analysis in different tissues and time points, genome sequencing, Southern blot analysis, and addition to catfish linkage

maps (Takano et al., 2008; Jiang et al., 2010; Liu et al., 2010a, 2010b; Liu et al., 2011c, 2011d; Niu et al., 2011). In this manner, we have integrated our functional genomics results with genome mapping and QTL analyses. Additional details will be gleaned from past functional genomics studies as the catfish transcriptome is better assembled and merged with the catfish whole genome sequence (now under assembly). Highly up- or downregulated transcripts with previously unknown identities may be annotated based on better assembly (i.e., connection of untranslated region ESTs with coding open reading frames) and/or by improved annotation of teleost-specific genes in public databases. Similarly, while gene ontology and gene pathway analyses have greatly enhanced microarray results from model species over the last decade, creation of teleost-specific ontologies and functional pathways are largely lacking outside the area of zebrafish developmental research. As fish cellular and whole-animal knockdown and knockout studies expand and results are compiled, microarray experiments and other large-scale gene expression profiling projects will become more fruitful in species such as catfish.

Next-Gen Perspective and Future Directions

The advances in next-generation sequencing seen over the last three years have opened the door for faster, cheaper, and more comprehensive functional genomics studies in aquaculture species. While prices for next-generation sequencing as of this writing still nominally exceed those of microarray analysis on a per sample basis, the equation is quickly changing with the widespread use of barcode tags for combining multiple samples into a single lane, decreasing reagent costs, increasing throughput, and the spread of next-generation platforms to individual laboratories. This last factor should particularly drive down costs as individual laboratories can conduct next-generation sequencing at cost rather than accepting the markups, delays, and higher labor costs of core or commercial service providers. In the small catfish genomics community, while previously depending on outside laboratories, PIs now have direct access to Illumina MiSeq and HiSeq 2000 instruments as well as a Roche 454 FLX. Several next-generation-based gene expression profiling experiments have been conducted over the last year in our laboratories, with several manuscripts now under preparation. These experiments include examination of mucosal immune responses in the catfish intestine following *E. ictaluri* infection, examination of the mucosal immune response in the catfish gill following *F. columnare* infection, and identification of sex-specific gene expression in catfish gonads. Data analysis techniques for RNA-seq data are still somewhat fluid with better assembly software having been released within the last 6 months. Our pilot studies have obtained good results using the analysis pathway illustrated in Figure 13.1 which combines several assembly software programs and utilizes catfish ESTs to form a comprehensive reference transcriptome. Short RNA-seq reads (100 bp) are then mapped to the reference and gene expression ratios calculated using CLC Genomics WorkBench (CLCbio, Aarhus, Denmark) software using the standard RPKM (reads per kilobase of exon model per million mapped reads) calculation. In analysis of expression in the catfish intestine, for example, we obtained an average contig size of 1108 bp for our RNA-seq contigs from the trans-Abyss-based assembly of approximately 195 million reads sequenced in a single lane of Illumina HiSeq 2000. A total of 176,481 contigs were obtained, representing approximately 20,000 unique genes as determined by BLASTX analysis. Gene expression analyses have identified 493 differentially expressed genes at 4 h following exposure to *E. ictaluri*, 659 genes at 24 h postexposure, and 829 genes at 3 days postexposure (Li et al., 2012). Analysis of gene lists from this experiment has revealed an intriguing combination of known

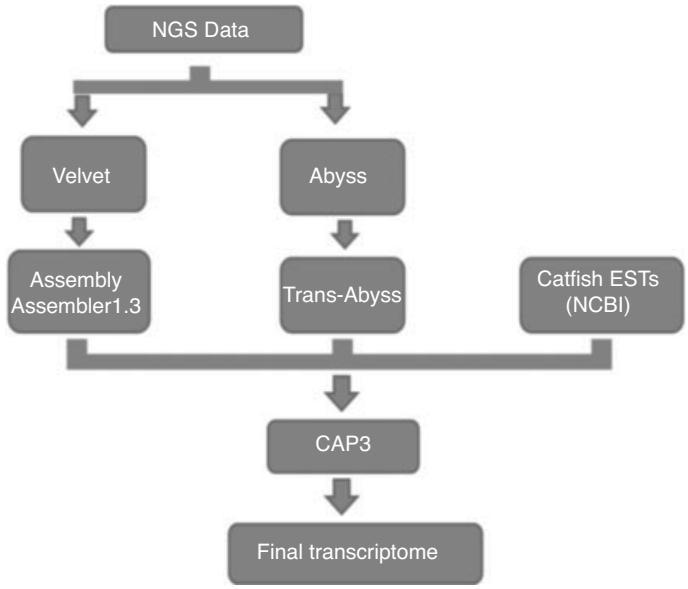

Figure 13.1 Assembly strategy for development of a comprehensive catfish reference transcriptome for RNA-seq analysis.

immune factors and previously unstudied genes involved in pathogen entry and replication in mammalian systems.

In catfish, the transition from microarrays to next-generation RNA-seq is still under trial. We are taking several factors into consideration during this transitional period. First, the catfish genomics community is small with only two main research groups. Costs for microarray updates, new-generation scanners, reagents, etc., therefore, cannot be disseminated among many users as is the case in larger species groups. Second, the catfish transcriptome, while largely captured by EST efforts, still continues to expand based on RNA-seq results, and is likely to be a moving target, although we believe that vast majority of the catfish transcriptome has been captured, until a high-quality reference genome assembly is obtained. Third, costs for RNA-seq continue to decline dramatically, with expected large decreases in the near-term as we transition from use of commercial providers to our own next-gen platforms and increase utilization of barcode tags. Fourth, RNA-seq approaches allow easy multiplexing of experimental aims (i.e., capture a tissue transcriptome, identify SNPs and microsatellites, and analyze gene expression in one experiment), which drives down real costs. Fifth, RNA-seq approaches, criteria for publication, data submission standards, assembly algorithms, and expression profiling algorithms are still very fluid, making it difficult to accurately budget time and resources for experiments. While the fast-moving nature of next-generation technology has been very beneficial, it is difficult to develop long-lasting protocols and test data repeatability in such a dynamic environment. Sixth, multiple RNA-seq projects can quickly result in laboratory bottlenecks as multiple users need to utilize computing resources to handle terabytes of data. Seventh, RNA-seq projects require training of individuals in basic programming and bioinformatics, transferring focus from bench experiments and/or field work to long-term data analysis and management. All of these considerations taken together have us leaning toward RNA-seq experiments, with the

utilitization of microarrays in the future for larger, long-term projects.

The transition to next-generation approaches for capturing and interrogating the transcriptome represent a fundamental change from bench science to informatic sciences. In our experience, time-consuming processes such as clone picking and large-scale plasmid preparations have been largely eliminated, but they have been replaced with data management and analysis chores. Data must be carefully and intelligently handled to prevent loss or swamping laboratory computer resources. New investments in server computers, supercomputer or cloud data storage and run time, and software licenses have been necessary in our laboratories. In spite of these changes, next-generation sequencing-based techniques hold tremendous promise for increasing the speed, quality, and possibilities of catfish functional genomics in the near future.

Research in catfish functional genomics to date has identified a number of putative biomarkers linked with different states of stress and/or disease resistance and susceptibility. Many of these biomarkers have additionally been added to catfish genetic maps as potential expression QTL. Our understanding of catfish immune responses and their similarities and differences with other model species has been considerably expanded. While funding for catfish genomics research is tight, decreasing assay costs and access to high-quality catfish transcriptome and genome sequences should speed the rate at which research is conducted and the breadth of traits that are investigated. As SNP-based whole genome association and QTL studies become common in catfish, functional genomics research will be critical to identifying and understanding the role of candidate genes for important performance and production traits.

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Chapter 14
Functional Genomics in Shrimp Disease Control

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Abstract: Over the past three decades, shrimp aquaculture has transformed from subsistence farming to a major industry due to the global increases in demand of seafood. As shrimp farming was intensified, diseases have become a major challenge to the industry worldwide, particularly those caused by viruses. To mitigate the need of the growing industry and to counter the threat of the emerging viral diseases, captive breeding programs have been developed in penaeid shrimp. In recent years, functional genomics have been used to complement the classical genetic approaches in developing virus-resistant shrimp lines. This chapter provides an overview of the current status of shrimp functional genomics toward developing markers for disease resistance, isolating differentially expressed genes and understanding the molecular mechanisms of viral pathogenesis in shrimp.

Introduction

Shrimp farming industry is just over 30 years old and estimated global production of farm-raised shrimp has already reached 3.36 million metric tons in 2007 with a value of over US $14 billion (FAO, 2009). Shrimp is the seafood item of highest demand in the United States, Japan, and the Europe, and in many tropical and subtropical countries, with a large coastal boundary; farm-raised shrimp represents one of the important agricultural commodities in terms of export earnings. The importance of this growing industry is reflected by the millions of people worldwide whose livelihood depends directly or indirectly on this industry.

Despite a major increase in global production of shrimp over the past three decades, shrimp farmers have experienced significant economic losses in recent years, primarily due to the outbreak of viral diseases. In fact, viral diseases are a major threat to the shrimp industry and periodic outbreaks of either existing or emerging viral diseases cause significant economic losses globally. For example, since their emergence, epizootics caused by infectious hypodermal and hematopoietic virus (IHHNV), white spot syndrome virus (WSSV), and yellow head virus (YHV) have resulted in economic losses that range in the billions of dollars worldwide (Lightner, 2005). In South America, Taura syndrome virus (TSV) has resulted in losses of more than $400 million per year (Brock et al., 1997; Dhar et al., 2004). Therefore, it would not be unreasonable to predict that the known and the emerging viral pathogens will continue to pose a serious challenge for the expansion and sustainability of shrimp industry worldwide.

Most shrimp viral diseases, such as those caused by IHHNV, TSV, and WSSV were spread regionally and globally through the movement of live shrimp stocks before their etiology and pathogenesis were well understood

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

and before the development of specific viral diagnostic protocols. In the aftermath of the viral pandemics, the industry moved rapidly to develop biosecurity programs to protect the industry and prevent further outbreaks. These efforts, as well as the establishment of integrated research programs dedicated to improving the health of seed stocks, are critical to sustain shrimp production globally.

In recent years, genomic approaches have been applied in developing genetic markers and assisting captive breeding programs of penaeid shrimp. Reviews covering the developments of shrimp breeding programs and functional genomics in penaeid shrimp have been published (Moss et al., 2005; Deoraj et al., 2006; Dhar et al., 2008; Cock et al., 2009; Robalino et al., 2009). The goal of this chapter is to highlight functional genomics approaches that have been used to study viral diseases of shrimp and not to serve as a comprehensive review of shrimp breeding programs or shrimp functional genomics.

Captive Breeding Programs

The foundation of captive breeding programs in shrimp was originally laid out in the early 1930s by Dr. Motosuka Fujinaga and his colleagues who carried out critical studies on the life cycle of marine shrimp. In the early 70s, the first commercial shrimp farming industry named Marifarms, Inc., was born in the state of Florida in the United States. During its existence during the period of 1968–1982, research at Marifarms was aimed to develop a captive breeding program with some success. In a recently published book entitled *Marifarms Incorporated: Memoirs of a Shrimp Farmer, The Story of How a Giant Industry Developed*, John R. Cheshire elegantly described the rise and fall of the first commercial shrimp farming industry in the world (Cheshire, 2005). In retrospect, the collapse of Marifarms was primarily due to the unavailability of an adequate number of postlarvae and poor growth of stocked postlarvae, which was most likely due to the outbreak of IHHNV. While the researchers at Marifarms tried to develop shrimp hatcheries to mitigate the shortage of supply of postlarvae, no attention was paid to what is now called as "biosecurity" (Lightner, 2005). In fact, viral diseases were not known in shrimp until 1974, when the first viral disease, baculovirus penae (BP), was described (Couch, 1974). The fall of Marifarms clearly led to the realization that in order to develop a sustainable shrimp industry, the development of a captive breeding program and preventing the introduction of pathogens in the hatcheries are a necessity. It was also realized that the use of wild caught postlarvae or broodstocks by farmers may pose a serious health risk to shrimp aquaculture, and therefore, the exclusion of specific pathogen needs to be an integral part of the shrimp-breeding program. Therefore, in the early 1980s, when the US Marine Shrimp Farming Program (USMSFP) was established in the Oceanic Institute (OI), Hawaii, improvement in growth and disease resistance became the primary focus of the shrimp-breeding program. The development of captive breeding programs and the biosecurity measures to contain the introduction of microbial pathogens in shrimp breeding program have been reviewed (Moss et al., 2005).

Specific Pathogen-Free (SPF) Shrimp Programs

The International Council for the Exploration of Sea (ICES) has developed a "Code of Practice to Reduce the Risks of Adverse Effects Arising from the Introduction of Non-Indigenous Marine Species" (Sindermann, 1990). In the wake of periodic outbreak of diseases caused by viruses such as TSV, WSSV, and YHV, the USMSFP used a modification of ICES guidelines to develop Specific Pathogen-Free (SPF) shrimp lines (Pruder et al., 1995). These guidelines recommend

that pathogens that could be reliably detected should be excluded from the facility engaged in SPF Programs. A working list of specific and excludable pathogens for penaeid shrimp has been developed, and the list is growing as new pathogens are emerging (Moss et al., 2001a).

A SPF breeding program consists of the following components: (1) identification of wild shrimp stocks, (2) evaluation of health status and disease history, (3) collection and testing of samples for specifically listed pathogens, (4) importation and quarantine of population (F_0) and monitoring F_0 stock, (5) production of an F_1 generation from F_0 stock, (6) destruction of F_0 stock, (7) culture of F_1 stock through critical stages, health monitoring, and testing for listed pathogens, and finally (8) if stocks deemed free of listed pathogens, the F_1 stock may be defined as SPF and released from quarantine. These stocks are referred to as "high health" when they leave the biosecure environment of quarantine (Moss et al., 2001b; Lightner, 2003).

The development of the captive breeding program in *Penaeus vannamei* shrimp by the USMSFP paved the way to the development of improved stocks through the processes of natural and artificial selection. As a result, currently nucleus breeding centers, broodstock providers, and even some shrimp farmers routinely breed the survivors of natural or induced disease outbreaks with the aim to develop specific pathogen-resistant (SPR) lines (Moss, 2005; Moss et al., 2005). As an example, SPR stocks of *P. stylirostris* resistant to IHHNV were developed by breeding the survivors of an IHHNV outbreak (Clifford, 1998). Similarly, in the aftermath of the TSV outbreak in the western hemisphere in 1992, the OI developed *P. vannamei* lines with varying levels of resistance to TSV (Moss et al., 2005). However, a downside of this approach was the negative correlation ($r = -0.15$, $p < 0.001$) observed between TSV resistance and shrimp growth. Compared to TSV-susceptible lines, TSV-resistant shrimp had a lower mean weight, molted less frequently, and had longer molt cycle (Moss et al., 2002). In a similar study (Perez et al., 2005), a negative correlation was reported between shrimp harvest weight from commercial farms and survival to WSSV. The estimated heritability of WSSV resistance was found to be extremely low (0.03–0.07), suggesting that WSSV resistance is polygenic. Currently, the challenge in developing resistant lines of shrimp against TSV, WSSV, and other viruses lies in finding stocks that contain resistant traits against these viruses.

Linkage Maps of Shrimp

In order to understand the genetic basis of resistance, susceptibility, and other heritable traits, efforts have been made to develop genetic linkage maps in shrimp. The first mapping families were developed for *P. vannamei* by the USMSFP (www.usmsfp.org) and for *P. monodon* and *P. japonicus* by the Australian Institute of Marine Sciences (AIMS; www.aims.gov.au). The estimated physical size of the shrimp genome is 2.3×10^9 bp, which corresponds to approximately 70% of the human genome size ($\sim 3 \times 10^9$ bp and 3000 cM) (Chow et al., 1990). The haploid chromosome numbers reported for penaeid shrimp vary from 43 to 46, depending on the species (Chow et al., 1990; Campos Ramos, 1996; Wilson et al., 2002; Li et al., 2003). Because of small chromosomes, karyotyping of shrimp has been difficult. However, recently, You et al. (2010) reported karyotyping of *P. monodon* chromosomes with a tentative assignment of the 44 pairs of *P. monodon* haploid chromosomes into 40 metacentric, 1 sub-metacentric and 3 acrocentric chromosomes. So far, only low-density linkage maps have been constructed for *P. monodon* (Wilson et al., 2002; Maneeruttanarungroj et al., 2006), *P. japonicus* (Li et al., 2003), *P. vannamei* (Zhang et al., 2006), and *F. chinensis* map (Li et al., 2006). The map of *P. monodon* was constructed using three mapping families and 116 amplified fragment length polymorphism markers. The map contained 8 major and 11 minor linkage groups

(http://www.aims.gov.au/shrimpmap); the average interval between adjacent markers was 22.4 cM with an estimated total genome length of 1412 cM. The male shrimp map contained 47 linkage groups, and the female shrimp map had 36 linkage groups (Wilson et al., 2002). There are approximately six markers per linkage group in both male and female maps with an average marker spacing of 11–13.5 cM. Clearly, more markers are needed to increase the map coverage in this species. The linkage map of *P. japonicus* has a higher resolution and better genome coverage (Li et al., 2003). The male map contained 43 linkage groups with genome coverage of 1781 cM, and the female map contained 31 linkage groups with genome coverage of 1026 cM. The average density of markers in both maps was approximately 1 per 10 cM, and more markers are needed to increase the resolution of both maps. Recently, a low-density linkage map has been developed for the *P. vannamei* (Zhang et al., 2006). There are 45 linkage groups in both male and female maps, and there are 7.1 and 5.9 markers per linkage group in male and female maps, respectively. The sex-linked markers found on the maternal linkage group *P. vannamei* indicated that the female is heterogametic (Zhang et al., 2006). Low-density linkage maps have also been published for *F. chinensis* (Li et al., 2006). There are 36 linkage groups in both male and female *F. chinensis* maps with approx. 6 markers per linkage group.

The confirmation of sex-linked markers on the maternal, but not paternal, map in *P. vannamei* (Zhang et al., 2006) highlights the potential of using the map-based information to develop all female lines in shrimp. Since females grow bigger than males in shrimp, all female families will have a significant impact on yield in shrimp aquaculture. In addition, different penaeid species differ in their susceptibility to different viruses. Therefore, as linkage maps are developed, determining the synteny (macro- and micro-colinearity of gene order) among various penaeid species will be very useful not only to expedite the development of high-density linkage maps but also to facilitate the development of marker-assisted breeding for disease resistance in penaeid shrimp.

DNA Markers for Disease Resistance in Shrimp

Random Amplified Polymorphic DNA(RAPD) Markers

The random amplified polymorphic DNA (RAPD) assay involves random amplification of DNA sequences using short oligonucleotides in a polymerase chain reaction (PCR). RAPD is a fast, cost-effective technique that needs no prior knowledge of the genome and has been widely used to detect polymorphisms for genetic mapping, phylogenetics, and population biology. Because of the existing paucity of genomic information in penaeid shrimp, the RAPD technique is quite suitable for genetic studies in this group. The RAPD approach has been used to identify population-specific markers in captive breeding program in *P. vannamei* (Garcia et al., 1994), to determine the genetic variation and population structure of wild populations of *P. monodon* shrimp (Klinbunga et al., 2001; Tassanakajon et al., 1998) and wild *P. stylirostris* (Aubert and Lightner, 2000). So far, there have been only two studies that employed RAPD technique to identify genetic markers against virus disease resistance in shrimp (Alcivar-Warren et al., 1997; Hizer et al., 2002). In one study, Alcivar-Warren et al. measured the levels of genetic diversity of high and low growth families of SPF populations of *P. vannamei* upon challenge with IHHNV and BP. These authors reported that families with high IHHNV prevalence had lower levels of RAPD polymorphisms and vice versa, although no such relationship was observed for BP. In another study, Hizer et al. compared the RAPD fingerprints of an IHHNV-resistant *P. stylirostris* line of shrimp to IHHNV-susceptible wild shrimp and

identified unique RAPD markers that were associated with IHHNV resistance/susceptibility in *P. stylirostris*. The mean IHHNV load in virus-resistant line was over 1000-fold less compared to the susceptible population (3.3×10^1 copies of IHHNV genome/ng of DNA vs. 4.2×10^4 of IHHNV genome/ng of DNA). These authors identified five RAPD markers that were found in the resistant line at a much higher prevalence (88–100%) compared to the IHHNV-susceptible population (0–5% prevalence; Figure 14.1). Conversely, three markers were found predominantly in the susceptible population (82–100% frequency) compared to the resistant population (0–19% frequency). These two studies clearly demonstrated the utility of the RAPD method in initial identification of genetic markers against viral diseases in shrimp. However, we must point out that due to the dominant nature of RAPD markers, they cannot be identified across lines, among different laboratories. Therefore, in the longer term, it is important to use sequence-tagged codominant markers.

Microsatellite Markers for Disease Resistance in Shrimp

Microsatellites are short, highly repetitive DNA sequences, generally two to eight nucleotides long, and are abundant in the genome. Microsatellites are also highly polymorphic, codominant markers that are inherited in a Mendelian fashion (Tautz, 1989; Wright and Bentzen, 1994). Since the first report of microsatellite sequences in *P. vannamei* shrimp (Garcia et al., 1994, 1995), microsatellite markers have been used to assess the genetic diversity of SPF populations of *P. vannamei* (Wolfus et al., 1997; Cruz et al., 2003) and evaluating the genetic variation and population structure of wild populations of *P. monodon* (Xu et al., 1999, 2001), *P. vannamei* (Valles-Jiminez et al., 2004), *P. schmitti* (Maggioni et al., 2003), and *P. setiferus* (Ball and Chapman, 2003). To date, only two studies have employed microsatellite markers to screen resistant/susceptible lines of shrimp against viral diseases. In one study, Xu et al. reported that a certain allele of a

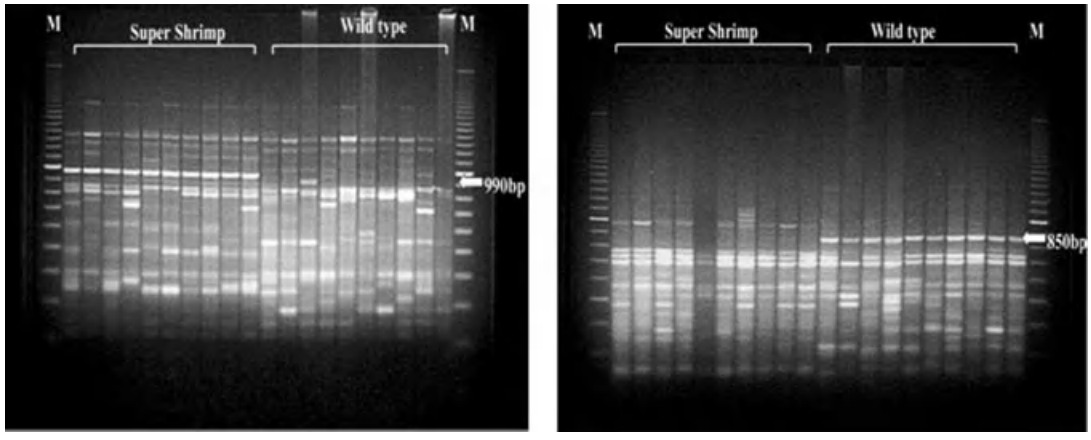

Figure 14.1 Identification of random-amplified polymorphic DNA markers for IHHNV resistance in *Penaeus stylirostris* shrimp. A 990 bp marker that is predominantly present in IHHNV-resistant SuperShrimp and an 850 bp marker that is predominantly present in IHHNV-susceptible shrimp are indicated by arrows. M = 100 bp DNA ladder of Invitrogen. (The figure is taken from Hizer et al. (2002) Genome 45:1–7 with publisher's approval.)

microsatellite, M1, is predominantly present in a TSV-resistant line (80% survivability) of *P. vannamei,* while some of the alternative allele is more prevalent in a TSV-susceptible line (Kona line, 15–20% survivability; Xu et al., 2003). In another study, Mukherjee and Mandal (2009) screened 42 microsatellites to identify potential marker(s) that can differentiate WSSV survivors from dead/moribund animals in shrimp populations where WSSV outbreak occurred. Only 1 out of 42 microsatellite markers could successfully differentiate the survivors from dead/moribund animals. One of the two alleles represented by this marker, a 71 bp allele, was predominantly present in the moribund/dead animals, indicating that this allele can be used to identify WSSV susceptible line of shrimp. These two studies demonstrated the feasibility of using microsatellite markers to identify virus resistant/susceptible lines in shrimp.

Differentially Expressed Genes as Candidate Markers for Disease Resistance in Shrimp

A major limitation in developing disease-resistant lines of shrimp has been the lack of availability of genetic resources containing disease-resistant traits in shrimp. Until very recently, there were only a handful studies that were devoted to understanding the molecular basis of viral pathogenesis in shrimp. The outbreaks of Taura syndrome disease in South America and white spot disease in Asia, and the rapid global spread of these diseases with massive economic losses forced researchers from both industry and academic institutions to pursue research to determine the genetic and molecular basis of virus resistance in shrimp. As a result, during the past five years, a series of studies have been conducted to identify candidate genes in shrimp that are differentially expressed between healthy and disease conditions for a number of viral diseases in shrimp. Identification of candidate genes involved viral pathogenesis is an important step toward development of markers for virus-resistant shrimp lines.

Considering the fact that the domestication process in shrimp has begun only recently and the genetic maps are in their infancy, reverse genetics approaches can expedite the discovery of genes that are associated with resistance and or susceptibility in shrimp. The association of the transcriptional activity of a gene to a specific biological process (e.g., resistance and or susceptibility), also known as transcriptome analysis, is performed using a variety of methods. In the following section, different methods that have been applied to identify gene(s) involved in different viral pathogenesis in shrimp are discussed.

Expressed Sequence Tag (EST) Analysis

The shrimp genome contains thousands of genes, the expression of which is temporally and spatially governed by a regulatory network. Modulation in this intricate regulatory network leads to a variety of developmental and pathological conditions. Therefore, isolating and characterizing genes that are differentially expressed under different biological conditions (e.g., healthy vs. diseased) are critical in understanding those biological processes.

Expressed sequence tags (ESTs) are typically single-pass sequences of randomly selected clones from a cDNA library that represents certain tissues or an organism under an experimental condition of interest. Since ESTs are generated by sequencing clones at random from a population of cDNA clones, the frequency of occurrence of a particular EST is largely determined by the level of abundance of the transcript in the tissue/organism from which it is derived. ESTs, therefore, reveal information pertaining to both transcript abundance and diversity. Because of the relatively low cost, and the ease of producing and analyzing sequence information, ESTs have

been generated from a number of *Penaeus* species under a variety of physiological conditions. Currently, over 196,000 EST sequences are available for shrimp (http://www.ncbi.org, http://www.marinegenomics.org) and a number of large-scale EST sequence projects for shrimp have been initiated (http://pmonodon.biotech.or.th, ShEST http://www.shrimp.ufscar.br).

The first report of ESTs in shrimp was published in 1999 to compare the gene expression patterns across tissues (i.e., cephalothorax, eyestalks, and pleopod) of black tiger shrimp, *P. monodon* (Lehnert et al., 1999). Subsequently, a number of papers were published that compared the transcriptome profiles of healthy and diseased (WSSV, YHV, TSV, and *Vibrio*-infected) shrimp (Rojtinnakorn et al., 2002; He et al., 2005; Pan et al., 2005; Zhao et al., 2007; Wang et al., 2009; Prapavorarat et al., 2010). Rojtinnakorn et al. (2002) compared the transcriptomes of healthy and WSSV-infected kuruma prawns (*P. japonicus*) derived from hemocyte cDNA libraries. The genes involved in defense response were found to be more expressed in WSSV-infected compared to healthy animals (15.7% in the WSSV-infected vs. 2.7% in the healthy animals). Among the immune genes, genes that encode protease inhibitors, apoptotic peptides, and tumor-related proteins were found only in the infected library and not in the healthy library, suggesting that these proteins may be involved in defense mechanisms in kuruma prawn (*P. japonicus*) against WSSV infection (Rojtinnakorn et al., 2002). Suppression subtractive hybridizations (SSH) were used to isolate the differentially expressed genes in the hepatopancreas and hemocytes of *P. japonicus* shrimp that survived WSSV challenge (He et al., 2005; Pan et al., 2005). Genes that showed similarity to interferon-like protein and (2′–5′) oligo (A) synthetase-like proteins, that are a part of the interferon pathways in vertebrates, were isolated from the hemocyte SSH library. Wang et al. (2006) isolated a C-type lectin, FcLec3, from *Fenneropenaeus chinensis* using EST analysis that was found to be upregulated during WSSV infection, suggesting that this protein may be involved in the innate immune response of shrimp against WSSV infection.

Zheng and Lu (2009) used crayfish (*Procambarus clarkia*) as a model species to study the crustacean immune response to WSSV infection. Using SSH and cDNA microarrays, these authors identified a set of differentially expressed genes that included serine protease inhibitors, chaperonin, synaptasome-associated protein of 25 kDa, tubulin, zinc-finger protein, intracellular fatty acid binding protein, extracellular superoxide dismutase precursor, arginine kinase, 70 kDa heat-shock-like protein, and Bax inhibitor-1. Homologues of many of these genes have also been identified from penaeid shrimp, suggesting a common antiviral mechanism in crustaceans.

EST analysis has also enabled the identification of many immune genes in shrimp that are involved in resistance against infection by RNA viruses such as YHV and TSV. Prapavorarat et al. (2010) compared the transcriptome profiles of healthy and YHV-infected *P. monodon* shrimp. A number of genes involved in cell defense and homeostasis and expressed during the early phase of YHV infection were identified. These include genes such as caspases, histidine triad nucleotide-binding protein 2, Rab11, b-integrin, tetraspanin, prostaglandin E synthase, transglutaminase, Kazal type serine proteinase inhibitor, and antimicrobial peptides. In addition, genes such as the anti-lipopolysaccharide factor isoform 6 (ALFPm6), crustin isoform 1 (crustinPm1), transglutaminase, and Kazal-type serine proteinase inhibitor isoform 2 (SPIPm2) were found to be upregulated upon YHV infection, indicating that these genes may be involved in anti-YHV immunity in shrimp.

In recent years, efforts have been made to use transcript abundance as a marker to develop disease-resistant line of shrimp. Lorgeril and colleagues, using a quantitative RT-PCR method, compared the abundance of anti-microbial peptide (AMP) (PEN2, PEN3,

Crustin, ALF, Lysozyme) and Cys-rich peptide genes between a nonselected shrimp line and a third-generation line of *P. stylirostris* shrimp selected for survival to natural *Vibrio penaeicida* infection. These authors reported that the expression of AMPs at 24 hours post-infection in selected line was significantly higher than that in the control line. The expression of PEN3 and lysozyme also differed between the two lines before *Vibrio* infection, making these two genes as predictive markers for resistance against *Vibrio in P. stylirostris* (Lorgeril et al., 2008).

Shrimp, like other invertebrates, rely on innate immunity for defense against microbial infection. The nonself recognition is activated by pattern recognition proteins (PRP) on microbial surfaces that are recognized by pattern recognition receptors. One such pattern recognition receptor is Toll-like receptors (TLR), a class of transmembrane glycoprotein. In *Drosophila*, TLRs was initially identified as key players in dorsal-ventral patterning and postembroynic development. Further studies revealed that TLRs play an important role in innate immunity in *Drosophila* (Leulier and Lemaitre, 2008). In mammals, the binding of PRPs to TLRs activates the transcription factors such as nuclear-factor κB and interferon regulatory factor 3, which in turn contribute to the expression of genes involved in antiviral and antibacterial immunity (Werling and Jungi, 2003; Takeuchi and Akira, 2007). Unlike mammals, in *Drosophila,* TLRs do not directly bind to PRPs directly and instead are activated by endogenous ligand Spatzel (Leulier and Lemaitre, 2008).

In shrimp, so far, TLRs have been identified from *P. monodon* (Arts et al., 2007), *Marsupenaeus japonicus* (Mekata et al., 2008), *F. chinensis* (Yang et al., 2008), and *P. vannamei* (Labreuche et al., 2009). We performed Bayesian phylogenetic analysis to assess the diversity of shrimp TLRs and their relationships to invertebrate and vertebrate homologues. The TLR sequences were grouped into three highly supported clades (Figure 14.2). One clade was composed of arthropod (moth, beetle, bee, fruit fly, and mosquito) and nematode TLRs. Shrimp TLRs formed a monophyletic cluster and showed close phylogenetic affinities to the third clade that contained marine invertebrates such as squid, horseshoe crab, scallop, sea urchin, zebra fish, and vertebrates such as chicken and human. Interestingly, this clade also included a couple of arthropod TLR sequences from silkworm and the fruit fly (Figure 14.2). This shows that shrimp TLRs are a group of closely related sequences that share closer affinities to marine invertebrates and vertebrates than to insect TLR's. The presence of a *Drosophila melanogaster* and a *Bombyx mori* TLR in two separate phylogenetic clades suggests that the common ancestor of the TLR genes arose before the split between insects and crustaceans. Information about the function of TLR in innate immunity in shrimp is limited and comes only from gene expression analysis. For example, in *P. monodon,* WSSV infection did not modulate TLR expression (Arts et al., 2007). In *M. japonicus,* TLR expression was upregulated upon injection of peptidoglycan but not poly I:C (Mekata et al., 2008). In *F. chinensis*, TLR expression was inducible after bacterial challenge, suggesting that the expression of the TLRs can be used as predictive markers for disease resistance in shrimp.

All these studies demonstrate the utility of generating ESTs in elucidating gene function in shrimp using reverse genetics approaches. The EST markers can then be used in marker-assisted breeding program for developing viral disease-resistant shrimp as well as in developing therapeutics against viral diseases in shrimp.

mRNA Differential Display

The mRNA differential display is a PCR-based RNA fingerprinting method that involves amplifying cDNA using arbitrary primers and separating the amplified cDNAs in denaturing polyacrylamide gel electrophoresis conditions. The method allows comparison of RNA

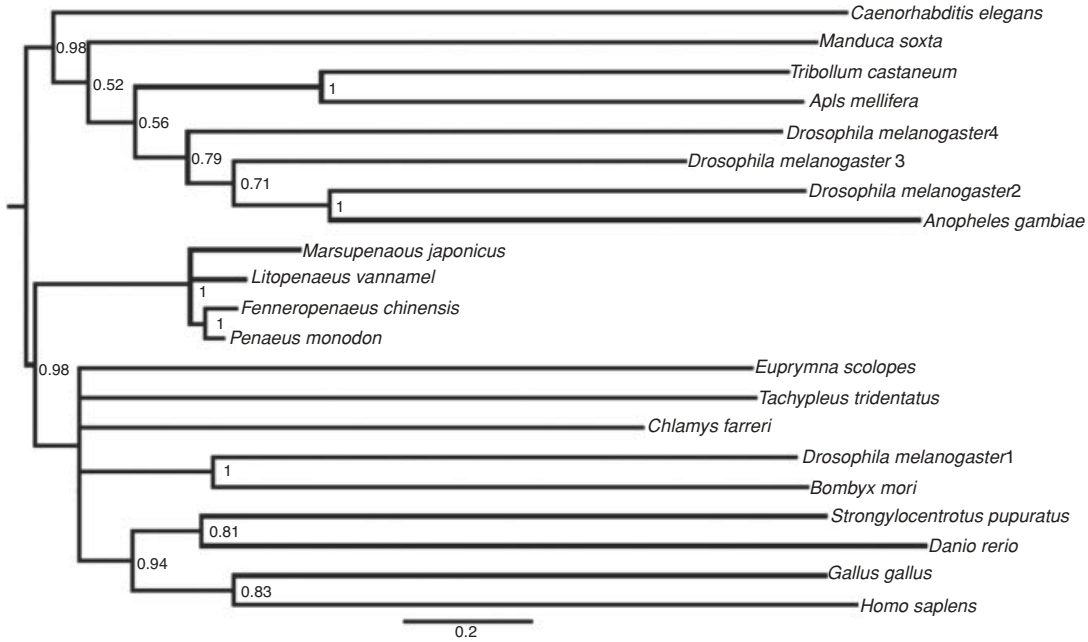

Figure 14.2 Bayesian phylogenetic tree of toll-like receptor genes from different species of shrimp, insects, and vertebrates. The toll-like receptor gene sequences were obtained from the GenBank database and a multiple alignment was performed using the MUSCLE algorithm (Edgar, 2004). The taxa included in the sequence alignment were *Fenneropenaeus chinensis* (EU433386), *Penaeus monodon* (EF117252), *Litopenaeus vannamei* (DQ923424), *Marsupenaeus japonicus* (AB385869), *Drosophila melanogaster1* (AF247766), *D. melanogaster2* (AY349654), *D. melanogaster3* (AF247767), *Apis mellifera* (XM_396158), *Anopheles gambiae* (AF444780), *Caenorhabditis elegans* (AF348166), *D. melanogaster4* (AF247769), *Strongylocentrotus purpuratus* (AF335484), *Chlamys farreri* (DQ350772), *Tachypleus tridentatus* (AB105059), *Bombyx mori* (AB070579), *Manduca sexta* (EF442782), *Euprymna scolopes* (AY956809), *Homo sapiens* (BC109094), *Gallus gallus* (AB046119.2), *Danio rerio* (BC107955), and *Tribolium castaneum* (XM_962703). The best-fit nucleotide substitution model, GTR+I+G, for the TLR sequence alignment was chosen according to the Aikake's Information Criterion using jModeltest (Posada, 2008). Bayesian inference phylogenetic analysis was performed using Mr. Bayes (Ronquist and Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) parameters included a run time of 2×10^6 generations, sampling every 1000 generations, and convergence acknowledged when the standard deviation of the split frequencies dropped below 0.01. Twenty-five percent of the sample trees were discarded as burnin. The 50% majority-rule consensus tree was visualized using Fig Tree v1.1.2 (Rambaut, 2008). Clade support was given as Bayesian posterior probabilities.

fingerprints of various conditions (e.g., healthy vs. diseased, resistant vs. susceptible) in parallel. Theoretically, 80 arbitrary primers in combination with three one-base (G/T/C) anchored oligo-dT primers (240 primer combinations) could detect 96% of the expressed genes in a cell ($[1–(0.96)^n]$, where n is the number of arbitrary primers; Liang and Pardee, 1997; Yang and Liang, 2004). The mRNA differential display using oligo-dT-anchored primers generally amplifies the 3′-end of the mRNA, and since the 3′-untranslated regions of mRNA species are very diverse, the nucleotide sequences of the differentially expressed cDNAs often do not show similarity with the GenBank database entries. However, the heterogeneity of the 3′-end of the mRNA allows identification of different members of a gene family.

The mRNA differential display method was used to identify differentially expressed genes in WSSV-infected shrimp (Astrofsky et al., 2002; Luo et al., 2003) and in *Vibrio*-infected *P. monodon.* While comparing the RNA expression profiles of healthy and WSSV-infected shrimp, Astrofsky et al. (2002) isolated several differentially expressed transcripts, including genes representing antimicrobial peptides, arginine kinase, and mitochondrial ATPase. However, less than 20% of the genes showed similarity with database entries. Luo et al. (2003) compared the RNA fingerprints of moribund and surviving *P. monodon* from shrimp farms in Fujian province, China, where WSSV outbreak occurred. These authors identified a novel gene, named *PmAV,* containing a C-type lectin domain from the surviving animals. A full-length clone of the homologous gene revealed that the cDNA contains carbohydrate recognition domain and there is a microsatellite repeat upstream of the TATA-box in this transcript. In vitro assay showed that the microsatellite repeat region modulates the promoter activity. It is unknown if this microsatellite modulates the expression of the gene in vivo during WSSV infection, and thus confer resistance against white spot syndrome disease in shrimp. Such a microsatellite in *PmAV* gene could serve as a potential marker for WSSV resistance/susceptibility.

Microarray Analysis

Microarray analysis is one of the most promising molecular genetics tools for gene exploration and functional gene mapping. The power of DNA microarray method lays not so much in their quantitative nature but rather in their ability to monitor the expression of thousands of genes simultaneously. The use of microarrays in shrimp aquaculture has been minimal, owing largely to the lack of commercially available microarrays containing shrimp sequences. However, this limitation can be overcome as more EST sequences become available. Genome-wide transcription profiling in shrimp should enable us to identify candidate genes that are involved in innate immunity against viral and bacterial pathogens, as well as genes that are essential to growth and development, reproduction, and metabolic and regulatory pathways.

There are only a handful of studies that employed microarray analysis to study viral pathogenesis in shrimp. Dhar et al. (2003) printed a small array of 100 elements to demonstrate the feasibility of employing microarray analysis to identify genes involved in WSSV pathogenesis. A total of 24 differentially expressed genes were identified, 16 of which were upregulated and 8 of which were downregulated. The expression profiles of three of the differentially expressed genes, an immune gene (C-type lectin-1), a structural gene (40S ribosomal protein), and a gene involved in lipid metabolism (fatty acid-binding protein) were further validated by real-time RT-PCR in healthy and WSSV-infected shrimp. This was the first such study to demonstrate the effectiveness of shrimp-based microarrays and how they might be exploited to study viral pathogenesis in shrimp.

Using cDNA microarray analysis, Wang et al. (2006) showed that immune-related genes such as heat-shock proteins (HSP70 and HSP90), trehalose-phosphate synthase, and ubiquitin C are differentially expressed during WSSV infection in *F. chinensis*. Robalino et al. (2007) studied the immune function of *Litopenaeus vannamei* shrimp using cDNA microarrays containing 2469 putative unigenes (http://www.marinegenomics.org) derived from EST from gills, hemocytes, and hepatopancreas after WSSV challenge. These authors identified the upregulation of a number of potential antimicrobial effectors such as anti-lipopolysaccharide factor, tachylectin-5A, lysozyme, and suppression of genes involved in protection from oxidative stress in WSSV-infected shrimp.

Global gene expression profiling by microarray analysis may also be applied to facilitate our understanding of molecular adaptations that allow some shrimp to tolerate viral

infection under certain environmental conditions. The biological insights that can be gained from transcription profiling have broad implications not only to identify molecular markers for disease resistance in shrimp breeding programs, but also to improve the health and welfare of shrimp and the quality of shrimp products.

RNAseq

Recent progress in DNA sequencing technology provides additional efficient ways for the study of differentially expressed genes. Among many of the newer technologies, RNAseq using next-generation sequencing such as Illumina's HiSeq 2000 sequencers can provide a robust snapshot of the entire transcriptome of an organism. However, RNAseq technology has not been applied in shrimp genomics studies, and there is no published report in this area yet. Because the next-generation sequencing generate shorter sequence tags than the traditional Sanger sequencing, accurate annotation and digital counting of sequence tags from specific genes may still be difficult, but it is clear that rapid progress is about to be made in this area.

Conclusions

Genomic research in shrimp has attracted significant attention in recent years primarily due to the global expansion of the shrimp industry and the emergence of viral diseases that are thwarting the industry growth. The lack of assured supply of genetically improved stocks and concerns over the use of unsustainable global resources (e.g., fishmeal and fish oil) in prepared diets are adding stress to the growth and development of shrimp farming. Genomic studies can significantly contribute to overcome these obstacles. The availability of genetic maps in shrimp and markers for disease resistance and other qualitative and quantitative traits will expedite the selective breeding programs for developing disease-resistant shrimp. Considering the fact that the natural resistance to viral diseases is infrequent in shrimp, effective protocols are also needed to detect resistance in populations when this trait occurs at a low frequency. Functional genomics approaches, although only recently applied in shrimp, have already provided important insights into the antiviral response mechanisms and the genes involved in antiviral immunity in shrimp. However, to address the disease resistance issues in shrimp in a comprehensive manner, there is now a growing need for a systematic characterization of shrimp genome. Finally, the development of genetically superior and disease-resistant lines of shrimp will greatly enhance the sustainability of this nascent industry worldwide.

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Chapter 15
Applications of Functional Genomics in Molluscs Aquaculture

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Abstract: The use of genomic tools in aquaculture is currently at a very early stage. Whole genome sequencing programs are being developed in several molluscan species. The genome of the Pacific oyster (*Crassostrea gigas*), which is one of the most important bivalve aquaculture species, is being sequenced using BAC clones. Moreover, genetic linkage maps have been published for ten molluscan species with the goal of finding genes associated with traits that affect production. Transcriptomic techniques are being used to study molluscan immunology to understand the molecular basis of their response to pathogens. The first molluscan DNA and oligo microarrays have been produced and used to study, among other subjects, the effects of pathogens and the impact of marine pollution. The application of genomic technologies may make significant contributions to numerous aspects of molluscan aquaculture, including disease resistance, diet design, reproduction, and growth enhancement.

Introduction

World bivalve production has increased significantly in recent years, from a combined total for wild catch and aquaculture of approximately 10.7 million tonnes in 1999 to 14 million tonnes in 2006 (FAO fishery statistics; Lee et al., 2008). Bivalve molluscs, specifically oysters, mussels, clams, and scallops, represent a significant portion of the world's fisheries production. These species have ideal characteristics for aquaculture because they do not require additional feeding beyond the food naturally present in seawater. Phytoplankton constitutes the majority of their diet, even when particles of other, nonliving material (detritus) may be consumed (Walne, 1974). Bivalves in the larval, juvenile, and adult stages can die from a variety of causes, which may be environmental or biological in origin. Physical and environmental factors can lead to mortality at each developmental stage; these factors include the following: prolonged periods of high or low temperatures, extreme salinities, particularly the low levels present after periods of heavy rain or melting snow, and industrial and domestic pollution, which can increase bacterial concentrations and organic and toxic material loads in seawater (Pipe and Coles, 1995; Braby and Somero, 2006; Carstensen et al., 2010). The main causes of mortality in bivalves are the diseases provoked by viruses, bacteria, and protozoa. Herpesvirus, iridovirus, and picornaviruses, among others, have been described as being responsible for viral mollusc diseases (Marteil, 1976; Comps and Bonami, 1977; Jones et al., 1996; Hine and Wesney, 1997; Renault and Novoa; 2004; Batista et al., 2007). Bacteria belonging to the

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.

genus *Vibrio* are the most important group involved in bivalve diseases, including brown ring disease and summer disease (Borrego et al., 1996; Paillard, 2004; Gómez-León et al., 2005; Huchette et al., 2006). Juvenile oyster disease, which was originally thought to be caused by some *Vibrio* spp and leads to significant mortality in *Crassostrea virginica*, seems to be caused by a proteobacteria (Boettcher et al., 2005). Species such as *Haplosporidium nelsoni* (Brown et al., 2004; Burrenson and Ford, 2004), *Bonamia ostreae*, *Marteilia refringens*, *Mycrocitos mackini,* and *M. roughley* or *Perkinsus marinus* (Carnegie et al., 2003; Berthe et al., 2004; Burrenson and Ford, 2004; Paillard, 2004; Villalba et al., 2004) are some examples of disease-causing protozoa. These microorganisms are able to cause significant mortality, which can decrease the global bivalve population and produce dramatic economic losses. The main groups susceptible to these diseases are bivalves in the larval and juvenile stages. Most aquaculture production relies on procuring juveniles from wild populations so that genomics could more easily be applied to those species that are produced in hatcheries, such as *Crassostrea gigas*, *C. virginica*, *Ruditapes decussatus*, *R. philippinarum,* and some pectinids.

Molluscs, in general, and bivalves in particular play an important role in ecosystems. Because they are filter feeders, molluscs link primary production with other organisms in the trophic chain. They can also act as reservoirs of elements, such as carbon and calcium, which are involved in shell construction. Moreover, because of their sessile nature, they are good indicators of pollution (Whitfield, 2001). Many bivalve species (oysters, clams, scallops, and mussels) have been commercialized as food or for other purposes (e.g., pearl oysters), which generates an added economic value. In spite of their economic importance, genomic studies of bivalves have lagged behind those of other species (Hedgecock et al., 2005; Saavedra and Bachère, 2006), and most of the literature dealing with bivalve genomics is focused on the effects of contaminants on these animals (Venier et al., 2003; Dondero et al., 2006; Venier et al., 2006).

Genomics focuses on the study of genes and their function. Its aim is to understand the structure of the genome, using techniques such as gene mapping and DNA sequencing to examine the molecular mechanisms regulating gene expression and understand the relationship between the genetic code and environmental factors in biological processes such as diseases. The study of genetic profiles induced by specific microorganisms will lead to the identification of genes involved in pathogen defense and the characterization of specific patterns that will help to identify resistance-associated genes. The use of genomic tools will facilitate the biological study of molluscs and increase our understanding of the biological processes that are critical for the success of molluscan aquaculture (Saavedra and Bachère, 2006).

Bivalve genome size (haploid content ranges from 5.4 pg to 0.65 pg) is intermediate between that of more primitive organisms, such as worms, and more highly evolved species, such as mammals (Gregory, 2004). The haploid number of chromosomes is between 10 and 23, with the size of the chromosomes being very homogeneous (Thiriot-Quiévreux, 1994). This characteristic makes it difficult to differentiate between chromosomes using conventional G- and C-banding; fluorescent in situ hybridization (FISH) is required to identify the chromosomes (Wang et al., 2005a, 2005b). The noncoding fraction of bivalve DNA contains a high number of satellite sequences and repetitive motifs. Curiously, it has been shown that one single repetitive motif present in high numbers in some bivalve species is also found in closely related species, reinforcing the library hypothesis, which suggests that some satellite sequences present in a group of organisms can spread randomly into the genome of other species (Nijman and Lenstra et al., 2001). FISH studies have revealed that bivalves are the only invertebrates that possess the vertebrate telomeric sequence, and some repetitive sequences seem

to be related to transposable elements (López-Flores et al., 2004).

Not as much is known about nuclear genes in bivalves compared with other aquatic species. Although mitochondrial DNA represents only a small fraction of total DNA, bivalve mitochondrial sequences are abundant in the databases because of their use in phylogenetic studies and population genetics research. Oysters (*Crassostrea* sp.), bay scallops (*Argopecten irradians*), and mussels (*Mytilus* sp.) are the species with the most available sequences in the databases. According to data gathered from the FAO (http://apps.fao.org/default.jsp), the Pacific oyster (*C. gigas*), Yesso scallop (*Patinopecten yessoensis*), and Manila clam (*Ruditapes phillipinarum*) are included in the "top 12" global aquaculture species. However, nuclear DNA sequences of both (Yesso scallop and Manila clam) are minimally represented in the databases (Saavedra and Bachère, 2006). In the last two decades, a collection of nuclear genes has been obtained as a result of traditional gene cloning research. Several genes have been cloned using a classical molecular approach due to their relevance for various aspects of biology, such as evolution, development, digestion, immunity, shell formation, and stress resistance. However, further studies using genomic approaches are needed to understand physiological processes such as growth, reproduction, and immunity, which are of great interest to aquaculture.

Sequencing Methods and Bivalve Genomes

For the last 30 years, two main approaches have been used to sequence genomes: the BAC-to-BAC method and whole genome shotgun sequencing. The former does not require any previous information about the DNA sequence, and it is also known as the *de novo* method. DNA is fragmented using chemical (restriction enzymes) or mechanic methods, and the shorter pieces are cloned into DNA vectors, usually bacterial artificial chromosomes (BACs) because of their ability to house long pieces of DNA (usual insert size is 100–350 kbp, but can be greater than 700 kbp) (Shizuya et al., 1992). DNA fragments purified from individual clones are individually sequenced and assembled. The shotgun method is often used for sequencing large genomes, although its assembly is complex and difficult, especially for repeated sequences (Venter et al., 1996; Venter et al., 2004). DNA is broken up randomly into numerous smaller fragments, which are sequenced using the chain termination method. Overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Bioinformatics programs are used to assemble all the overlapping reads into a continuous sequence. Depending on the size of the gap between contiguous fragments, different techniques can be used to find the missing sequence. If the gap is small (5–20 kb), then polymerase chain reaction (PCR) can be used to amplify the region for sequencing. If the gap is large (>20 kb), then the large fragment is cloned into BACs. Although the shotgun method represented a significant advancement in DNA sequencing, new techniques have been surfacing in the last few years known as next-generation sequencing (see related chapters in this book). These technologies allow sequencing of millions of reads in a relatively short time (Voelkerding et al., 2009). The assembly and sequencing analysis, as well as the whole process, require significant computational and economic investment, but the high volume of data allows a full genome to be sequenced in a relatively short time. Several different platforms are available, including 454 Life Sciences (Roche), Solexa (Illumina), Solid (Applied Biosystems), and single molecule sequencing (Helicos Biosciences). All platforms share a common approach, involving massive parallel sequencing of DNA molecules that are spatially separated. (Brenner et al., 2000) Sequencing is performed through repeated cycles of polymerase-mediated nucleotide

extension (Voelkerding et al., 2009) or ligation reactions.

The new sequencing methods facilitate the sequencing of the genome of organisms of interest to both basic and applied research. The generated sequences are stored in public databases with free online access using bioinformatics tools. The National Centre for Biotechnology Information database is the most used (http://www.ncbi.nlm.nih.org). Other important databases for genomic sequence storage are the Genomes OnLine Database (http:/www.genomesonline.org) and the European Bioinformatic Institute (EBI) database (http://www.ebi.ac.uk/Databases/).

In molluscs, BAC libraries have only been reported for two commercial oyster species, eastern and Pacific (Cunningham et al., 2006), and Zhikong scallops, *Chlamys farreri* (Zhang et al., 2008). A total of 73,728 and 55,296 clones were picked and arrayed for *C. gigas* and *C. virginica*, respectively. The oyster BAC libraries are publicly available to the research community on a cost-recovery basis at www.genome.clemson.edu. The results from the BAC scallop library were a combination of the data from two BAC libraries generated from nuclear DNA, digested with two different restriction enzymes. The two libraries produced 73,728 and 7680 clones, respectively, and the combined libraries contained a total of 81,408 BAC clones. A previous library had been created in scallops using fosmids as vectors (Zhang et al., 2007), but the insert sizes were much larger in the BAC libraries (113 kb compared to 40 kb) and deeper genome coverage (9.1X to 4.3X) was obtained, suggesting that BAC libraries are a better tool for gene localization. Although many expressed sequence tag (EST) collections have been generated in different bivalve species, such as mussels (Bultelle et al., 2002; Marin et al., 2003; Venier et al., 2003, 2006, 2009; Brown et al., 2006; Pallavicini et al., 2008; Tanguy et al., 2008; Boutet et al., 2009), oysters (Meistertzheim et al., 2007; Tanguy et al., 2008; Green et al., 2009; Joseph et al., 2010), and clams (Gestal et al., 2007; Perrigault et al., 2009; Prado-Alvarez et al., 2009), characterization of these species on a DNA level has been limited (Saavedra and Bachère, 2006). Five bivalve species have been selected for genome sequencing: the scallop *A. irradians* (project ID 13038), *C. virginica* (project ID 12979), *Spisula solidissima*, (project ID 12959), *Pinctada maxima* (project ID: 41527), and the mussel *Mytilus californianus* (http://www.jgi.doe.gov).

Functional Genomics

Functional genomics uses all the information generated by genomic projects to describe gene functions and interactions. Gibson and Muse (2002) defined the field as "approaches under development to ascertain the biochemical, cellular, and/or physiological properties of each and every gene product," while Pevsner (2009) included the study of nongenic elements in his definition: "the genome-wide study of the function of DNA (including genes and nongenic elements), as well as the nucleic acid and protein products encoded by DNA." This discipline includes transcriptomics (the study of the whole set of transcripts) and proteomics (the study of the whole set of proteins encoded by a genome). Using the transcriptome, gene profiles may be analyzed in different tissues, organs, or experimental conditions. A remarkable example was described by Jackson et al. (2006), who used these molecular tools to discover new information about an important process in mollusc biology: shell formation. However, because of the economic importance of molluscs in aquaculture, most studies have been focused on analyzing the immune system with the aim of identifying the molecular basis of the most common pathologies (Saavedra et al., 2009).

Transcriptomic Studies: EST Databases

Although the number of bivalve nuclear genes available in the databases is quite limited,

several EST collections from cDNA or suppression subtractive hybridization (SSH) libraries have been generated in the last few decades with the aim of identifying protein-coding genes that are expressed within a tissue or in response to physiological disturbances. This is the part of the genome that is actually expressed and provides tools for further experiments, including microarrays, gene fishing, and rapid amplification of cDNA ends, among others. EST collections are the main source of genomic data in nonmodel organisms and are usually the first step for genomic projects. Many of the EST libraries were constructed from stimulated or infected animals to identify genes putatively related to the immune system. Consortia of several laboratories have helped to produce these kinds of data, such as the Marine Genomics project in America (www.marinegenomics.org) or the Marine Genomics Europe Network of Excellence (www.marine-genomics-europe.org) (Saavedra and Bachère, 2006). With the single exception of the invasive zebra mussel, *Dreissena polymorpha*, all the collections focused on species of commercial interest. These libraries have provided sequences that show homology with others that have already been described in different organisms. However, a significant number of generated ESTs did not show similarity to known genes using a BLAST search against the available databases.

Mussel ESTs

Since the mid-1970s, mussels have been widely used as bio-indicators because of their ubiquitous distribution, sessile adult condition, filtering activity, and plasticity in response to environmental changes (Goldberg and Bertine, 2000). Nevertheless, information about mussel genes was nonexistent until 2002. The first libraries were generated in 2002 from the invasive zebra mussel, *D. polymorpha*; differential display-PCR and subtractive libraries were used to analyze the differential expression of mussel genes after exposure to several xenobiotics (Bultelle et al., 2002). In 2003, a new cDNA library was created in the Mediterranean mussel, *Mytilus. galloprovincialis*, by Venier et al. (2003) using cDNA from the hemolymph, gills, digestive gland, foot, adductor muscle, and mantle from unstressed mussels. A total of 524 clusters from 829 ESTs were analyzed. Most of the sequences did not shown known homology because of the lack of available mussel sequences in the databases, although some ESTs with homology to proteins involved in the cytoskeleton and respiratory chain were found. A small number of ESTs showed homology with immune-related genes such as the β-glucan binding protein, LPS-binding protein, and some proteasome components, lectins and transcripts with homology to antimicrobial peptides. These ESTs were combined with other cDNA and subtracted libraries (Pallavicini et al., 2008) from bacteria-stimulated mussels and, in 2009, Venier et al. (2009) constructed, sequenced, and annotated 17 cDNA libraries from different *M. galloprovincialis* tissues: gills, digestive gland, foot, anterior and posterior adductor muscle, mantle, and hemocytes. A total of 24,939 clones were sequenced from these libraries, generating 18,788 high-quality ESTs that were assembled into 2446 overlapping clusters and 4666 singletons, resulting in a total of 7112 nonredundant sequences. Bioinformatic screening of the nonredundant *M. galloprovincialis* sequences identified 159 microsatellite-containing ESTs. Clusters, consensuses and related similarity, and gene ontology searches were organized in a searchable database known as MytiBase (http://mussel.cribi.unipd.it). An SSH and a nylon macroarray were used by Brown et al. (2006) to assess the effect of exposure to benzo[a]pyrene in blue mussels, *Mytilus edulis* (Brown et al., 2006). The upregulated transcripts were involved in oxidative stress, cell adhesion, and metabolic processes. Another SSH library was also generated in the zebra mussel from lipopolysaccharide-, peptidoglycan-, and

zymosan-stimulated hemocytes. A total of 44 ESTs, most of them without putative functions, were differentially expressed. Immune-related molecules such as matrilin or agglutinin were also upregulated (Xu and Faisal, 2007). The European Network of Marine Genomics used two species of mussels, *M. edulis* and *Bathymodiolus azoricus*, to construct a normalized cDNA library using different tissues and physiological conditions (Tanguy et al., 2008). A total of 60 and 362 contigs and 1918 and 2865 singletons were identified in *B. azoricus* and *M. edulis*, respectively. Stress-related genes, and genes involved in metallic and oxidative stress responses, were identified. In the triangle snail mussel (*Hyriopsis cumingii*), more than 5000 ESTs were sequenced, and 481 contigs and 1165 singletons were identified. BLAST similarity analysis indicated that almost half (46.5%) of these ESTs were homologues of known genes. A total of 201 microsatellites were identified from these ESTs, with 31 having sufficient flanking sequences for primer design (Bai et al., 2009). Transcripts with homology to heat-shock proteins, ferritins, antimicrobial peptides, lectins, and stress-related genes were found.

The previous EST collections were created using classical approaches such as cloning and Sanger sequencing strategies. However, in the last few years, several works using the pyrosequencing method (Margulies et al., 2005) in molluscs have been published, an approach that allows faster sequencing output and increased coverage. The first study was conducted in *M. galloprovincialis* and used total RNA from the digestive gland, foot, gills, and mantle; more than 175,547 reads were sequenced and grouped into a total of 8536 contigs (Craft et al., 2010). With the aim of studying innate immunity in the deep-sea mussel, high-throughput sequence analysis was carried out in the vent mussel, *B. azoricus*, using pyrosequencing technology (Bettencourt et al., 2010) to produce more than 700,000 sequencing reads.

Oyster ESTs

Because of the commercial and ecological importance of the oyster (*C. gigas* and *C. virginica*), the Oyster Genome Consortium was formed for the development of genetic and genomic tools. Apart from the BAC libraries for *C. gigas* and *C. virginica* (Cunningham et al., 2006) that have been previously described in this chapter, the availability of oyster DNA sequence information has been growing recently, with several groups reporting EST collections for *C. gigas* and *C. virginica*. The first published libraries were based on the eastern oyster, *C. virginica*, and generated from hemocytes and embryos with the aim of identifying genes potentially involved in the stress response for use as bioindicators of exposure to environmental pollutants and toxic and infectious agents (Jenny et al., 2002). A total of almost 1000 ESTs were sequenced, approximately 40% of which were novel sequences. ESTs with homology to C-type lectin and scavenger receptors, proteinases and their inhibitors, metallothionein, precerebellin, collectins, and antimicrobial peptides were identified. An abundant number of genes related to DNA replication were found in an embryonic library. A similar study was conducted in the Pacific oyster, *C. gigas* (Gueguen et al., 2003), after exposure to four strains of *Vibrio* sp. A public database containing all the information is available online at http://www.ifremer.fr/GigasBase. A total of 55 sequences (grouped into 20 clone clusters) out of 1142 ESTs analyzed were classified as having immune function based on sequence homology with immune genes characterized in other organisms. Partially similar results to those obtained by Jenny et al. (2002) were found. Proteases, inhibitors of proteases, and adhesive proteins were highly expressed. However, no genes encoding antimicrobial peptides were detected, although ESTs homologous to molecules of the Rel/NF-κB signal transduction pathway were

expressed. An SSH library was generated in *C. gigas* to characterize up- and downregulated genes during hydrocarbon exposure. Genes involved in oxidative stress and in detoxification were overexpressed (Boutet et al., 2004). The same technique was used by Huvet et al. (2004) to identify genes in *C. gigas* that were differentially regulated between F2 progeny that were resistant and susceptible to the summer mortality associated with juveniles. A cDNA library was created in *C. virginica*, and more than 4000 ESTs were generated (Peatman et al., 2004). The most remarkable result was the detection of several transcripts with homology to heat-shock proteins and other proteins involved in stress responses and the identification of the antimicrobial peptide defensin B. A study of genes expressed in response to *P. marinus* challenge was carried out in the hemocytes and gills from both *C. virginica* and *C. gigas* (Tanguy et al., 2004). The construction of a cDNA library in bacteriophages allowed the identification of two different types of ferritins in *C. gigas* that have an important role in iron regulation (Durand et al., 2004). Genes involved in the immune system, cell communication, protein regulation and transcription, cell cycle, respiratory chain, and cytoskeleton were detected, and their expression was screened, showing an overexpression in challenged oysters. Even when some ESTs showed homology with immune-related genes, such as lectins or toll-like receptor 4 (TLR-4), the number of potential immune genes was not very high. One year later, Tanguy et al. (2005) developed another SSH library with the aim of identifying genes modulated after exposure to pesticides in *C. virginica*. Most of the identified genes were involved in the detoxification process, metabolism, and transcription. The molecular response to hypoxia stress in the gills, mantle, and digestive gland was also studied in *C. virginica* using an SSH library (David et al., 2005). The most highly modulated genes were involved in carbohydrate and lipid metabolism and in stress responses. A small percentage of potential immune genes were also found in ESTs identified in libraries from *C. gigas* affected by summer mortality. However, this technology led to the identification of antimicrobial peptides belonging to the mussel defensin family in oysters (Peatman et al., 2004; Gueguen et al., 2006). Cg-def (*C. gigas* defensin) was active against gram-positive bacteria *in vitro*, although its activity against gram-negative bacteria and fungi was either limited or not detected. With the aim of investigating the effects of domestic sewage on gene expression in *C. gigas*, a SSH was constructed from gills (Medeiros et al., 2008). A total of 15 genes were identified, which were related to biotransformation, membrane transport, aerobic metabolism, and the translational machinery. A total of 374 contigs and 5528 singletons were reported in *C. gigas* after the construction of a normalized cDNA library (Tanguy et al., 2008). Different ESTs with homology to extracellular receptors involved in growth, metabolism, and reproduction were identified. Some members of the P450 family and sequences with homology to the p53 tumor suppressor were also detected. Roberts et al. (2009) reported the construction of a library from hemocytes of *C. gigas* in which 2198 ESTs of at least 100 bp were generated and analyzed. Several genes detected in the library were significantly upregulated by bacterial challenge, including interleukin-17, astacin, cystatin B, the EP4 receptor for prostaglandin E, the ectodysplasin receptor, c-jun, and the p100 subunit of nuclear factor-κB. Fleury et al. (2009) conducted three large-scale cDNA sequencing projects in the Pacific oyster, *C. gigas*; the large number of generated ESTs was subsequently compiled in a publicly accessible database. A total of 40,845 high-quality ESTs that identify 29,745 unique transcribed sequences, consisting of 7940 contigs and 21,805 singletons were obtained. These new sequences, together with existing public sequence data, have been compiled into a publicly

available website at http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html. A total of 208 *in silico* microsatellites from the ESTs, with 173 having sufficient flanking sequence for primer design, were identified. Also, a total of 7530 putative, *in silico* single-nucleotide polymorphisms were identified using existing and newly generated EST resources for the Pacific oyster. A cDNA library was created in pearl oysters, *Pinctada martensii*, from adult tissues (Wang et al., 2011). A total of 7128 ESTs were sequenced and 788 contigs and 1351 singletons were identified. Although most of the found genes were related to cellular structure, protein binding, and metabolic processes, some putative immune genes, such as C-type lectins, ferritins, proteases, scavenger receptors, or heat shock proteins, were also identified.

Several types of genetic markers are available for genetic and genomic analysis, including allozymes, restriction fragment length polymorphisms, random amplified polymorphic DNA, amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) (Karl and Avise, 1992; Sekino et al., 2003; Yu and Guo, 2003; Liu and Cordes, 2004). In aquaculture species, AFLPs and SSRs are two of the most popular markers for genomic mapping (Kocher et al., 1998; Young et al., 1998; Yu and Guo, 2003). SSRs or microsatellites are tandem repeats of short sequences of one to six base pairs that are abundant in all eukaryotic genomes. They are codominant, highly polymorphic, and ideal for mapping studies. Traditionally, SSR markers are developed by cloning and sequencing from enriched genomic libraries, a process that is complicated and costly. However currently, ESTs provide a new source for the development of SSR markers. This methodology was developed in the eastern oyster, *C. virginica*. Screening of 9101 ESTs identified 127 (1.4%) SSR-containing sequences (Wang and Guo, 2007). In *C. virginica*, a normalized cDNA library was constructed from pooled RNA isolated from hemocytes, mantle, gill, gonad and digestive tract, muscle, and a whole juvenile oyster (Quilang et al., 2007), identifying a total of 35 microsatellites and more than 6000 putative SNPs. A total of 6528 clones were sequenced from this library, generating 5542 high-quality EST sequences representing 4688 unique sequences. These genomic resources provide tools and material for future microarray development, genetic linkage, and quantitative trait loci analysis. In *C. gigas*, a total of 147 microsatellite-containing ESTs were detected from more than 4000 ESTs in GenBank. Repeat motifs of two nucleotides were the most abundant type and these results complemented the SSR markers and will facilitate comparative mapping, marker selection, and evolutionary studies (Yu and Li, 2008). Moreover, another set of EST-SSR markers was developed in *C. gigas* bioinformatically, and a total of 17 polymorphic microsatellite markers were characterized (Wang et al., 2008). Tanguy et al. (2008) found microsatellite-containing ESTs in four bivalve species (*C. gigas, M. edulis, R. decussatus,* and *Bathymodiolus azoricus*), although their representation was less than 0.5% of all analyzed ESTs. Moreover, Taris et al. (2009) used microarrays and cDNA-AFLPs to identify ESTs differentially expressed in response to bacterial challenge in two different *C. gigas* families (heat-shock tolerant and heat shock sensitive). They reported that most of the differences in the transcription patterns of immune and metabolic genes between the tolerant and sensitive families can be attributed to constitutive differences in gene transcription.

The pyrosequencing method was also used in oysters. The first instance was described in the pearl-producing oyster, *Pinctada margaritifera*. A total of 276,738 reads were reported and 82 biomineralization-related proteins were identified (Joubert et al., 2010).

Scallop ESTs

The first scallop EST library was created in the bay scallop, *A. irradians*, in order to understand the factors that contribute to the growth

and development of the scallop adductor muscle (Roberts and Goetz, 2003). Of the genes identified based on sequence similarity, 33% were involved in cell structure or the cytoskeleton. A cDNA library was generated from the whole bodies (except for the digestive tracts and the intestines) of 10 Zhikong scallops, *Chlamys farreri* (Song et al., 2006). Almost 5000 ESTs were sequenced and grouped into a total of 637 contigs and 2142 singletons. ESTs were identified with homology to lectins, defensins, proteases and protease-inhibitors, heat-shock proteins, antioxidants, and Toll-like receptor gene homologues. The same group constructed another cDNA library from the whole bodies of 10 scallops stimulated with *Listonella anguillarum* (Wang et al., 2009). More than 5000 ESTs were analyzed, and most of them showed homology to genes involved in metabolic processes. A fosmid library was constructed in the same species to provide a preliminary assessment of the genome. The library consisted of 133,851 clones, and end-sequencing of 480 individual clones generated 828 sequences after trimming, with an average sequence length of 624 bp (Zhang et al., 2007). In the marine scallop, *Argopecten purpuratus*, a cDNA library was made from gonad tissue (Boutet et al., 2008). More than 400 unique genes were characterized, and most of them (more than 80%) did not show any known homology. However, genes related to development were upregulated, including testis-specific serine/threonine-protein kinase, vitellogenin, spermatogenesis and centriole-associated 1, calcineurin A, centrin, RNA-specific adenosine deaminase, and cytidine deaminase.

Clam ESTs

The Manila clam, *R. philippinarum*, was the first clam species in which a cDNA library was generated, in this case from a *Perkinsus olseni*-infected individual. A total of 1850 clones were sequenced, and 29 ESTs were associated with functional immune genes such as C-type lectin, lysozyme, and cystatin B (Kang et al., 2006). Two subtractive libraries were constructed in carpet shell clams (*R. decussatus*) after stimulation with dead bacteria (Gestal et al., 2007) or with *P. olseni* (Prado-Alvarez et al., 2009). Tanguy et al. (2008) reported a total of 124 contigs and 1814 singletons for *R. decussatus*, including immune genes with homology to lectins and ferritins.

Microarrays

A significant number of ESTs have been generated in bivalve molluscs through the construction of numerous libraries from different stimulated tissues. These collections have proven a valuable source of information because they contain genes whose expression is modulated in response to environmental, chemical, or biological stimuli. These ESTs can be used as probes in microarrays to hybridize other cDNA targets under different stringency conditions. DNA microarray technology enables the measurement of the expression of a large set of known genes or sequences. DNA molecules, ranging from oligonucleotides to complete cDNAs representing specific transcripts, are fixed onto a solid support. Because each array can contain thousands of probes, every hybridization reaction can accomplish many genetic tests in parallel. Microarrays have various applications, including the analysis of expression profiles, the detection of SNPs, genotyping analysis, and checking for mutations.

Mussel Microarrays

The first study using microarrays was conducted by Dondero et al. (2006) in the Mediterranean mussel, *M. galloprovincialis*. The methodology used was very different from current microarrays. A low-density oligonucleotide microarray known as Mytox-chip was used, which displayed a total of 24 mussel genes, including both normalizing elements and stress response-related genes, each represented on the array by one or two different 50-mer oligonucleotide

probes spotted in replicated samples on activated glass slides. The microarray was enriched for genes involved in pollutant and xenobiotic responses, and it was tested on mussels exposed to sublethal concentrations of mercury or a crude North Sea oil mixture. The same research group developed a new microarray known as MytArray 1.0, which included 1714 probes (76% singletons, approximately 50% putatively identified transcripts) plus unrelated controls from the gills, digestive glands, foot/adductor muscles/ligaments, and mantle/gonads of mussels of mixed sex that had been stimulated with water-soluble metal salts or mixed organic chemicals (Venier et al., 2006). After laboratory treatments with environmental pollutants, bacterial antigens, and viral-like polynucleotides, 18,788 high-quality ESTs from *M. galloprovincialis* were organized into a structured collection of 7112 transcript sequences named Mytibase (Venier et al., 2009). Using a cross-search strategy, 1820 putatively immune-related sequences were selected for use as oligonucleotide probes to define a microarray. This microarray was tested with RNAs from mussels injected with *Vibrio splendidus* (Venier et al., 2011). In order to assess the potential use of this molecular biology tool to analyze gene expression, different tissues and groups of mussels were analyzed. For example, this microarray was used to evaluate gene expression changes in the digestive gland of mussels fed with nutrients contaminated with okadaic acid (Manfrin et al., 2010). Another oligonucleotide-microarray was developed with 8874 probes representing 4488 different genes that recognized mRNAs from both *M. galloprovincialis* and *M. trossulus*; this array was used to analyze the effects of heat stress on gene expression (Lockwood et al., 2010). The same microarray was used to study the transcriptional responses of the two species to an acute decrease in salinity (Lockwood and Somero, 2011). Microarrays are also available for noncommercial species, such as the zebra mussel, *D. polymorpha*, for which a cDNA microarray (ZMB), including 716 genes generated from a foot byssus SSH cDNA library, was printed and used to compare gene expression during adhesion and nonadhesion (Xu and Faisal, 2009b). This microarray was also used to study gene expression profiles in different stages of byssogenesis (Xu and Faisal, 2010a).

Oyster Microarrays

To maximize the utility of all the oyster gene collections, a cDNA microarray was constructed based on EST libraries from several different tissues of *C. gigas* and *C. virginica* exposed to hypoxia, pesticides, bacterial and protozoan infections, hydrocarbons, hyperthermia, and summer mortality conditions (Jenny et al., 2007). A total of 5586 sequences from *C. virginica* and 3961 from *C. gigas* were initially collected from different SSH and cDNA libraries and submitted to Gene Ontology for annotation. The microarray slide consists of two complete side-by-side arrays, with each array containing duplicate spots for 4460 and 2320 amplicons for *C. virginica* and *C. gigas,* respectively. This cDNA microarray is publicly available to the research community and has already been used to identify candidate genes involved in stress tolerance in *C. gigas* by comparing families resistant and susceptible to summer disease (Lang et al., 2009; Fleury et al., 2010) and transcriptomic profiling of selectively bred Pacific oyster *C. gigas* families that differ in their tolerance to heat shock (Jenny et al., 2007; Lang et al., 2009). An *in situ* oligonucleotide microarray was developed using existing ESTs to analyze gene expression profiles during dermo disease caused by *P. marinus* (Wang et al., 2011). This array was also used to analyze the relationships between gene expression and a suite of environmental parameters (natural and anthropogenic) in the eastern oyster (Chapman et al., 2011). Given that quantitative PCR is usually used to validate microarray analyses, it is worth mentioning the report by Taris et al. (2008), highlighting the potential difficulties for using Q-PCR as a validation tool due to the presence of sequence polymorphism in oysters. They emphasize the need for extreme caution and thorough primer testing when

assaying genetically diverse biological materials such as Pacific oysters. Their findings suggest that melt-curve analysis alone may not be sufficient as a means of identifying acceptable Q-PCR primers. Minimally, testing numerous primer pairs seems to be necessary to avoid false conclusions from flawed Q-PCR assays for which sequence variation among individuals produces artifacts and unreliable quantitative results.

Proteomics

Proteomics is the large-scale study of proteins, particularly their structures and functions. The word "proteome" refers to all the proteins produced by an organism. While the genome is a rather constant entity, the proteome differs from cell to cell and it is constantly changing due to its biochemical interactions with the genome and the environment. Unlike the classical molecular biology dogma "one gene, one protein," the completion of the human genome project has revealed that there are far fewer protein-coding genes in the human genome that there are proteins in the human proteome. The large increase in protein diversity is thought to be due to alternative splicing and post-translational modification of proteins by processes such as photolytic processing, glycosylation, and complex protein formations. Moreover, correct three-dimensional folding is required for the protein to be in an active form. There are different branches in proteomics, such as protein separation, in which techniques are designed to separate individual proteins from a complex mixture, and protein identification, which involves protein sequencing. The most classical sequencing method is the Edman degradation although there are other techniques based on mass spectrometry, such as MALDI-TOF (matrix-assisted laser desorption/ionization) or the use of antibodies to recognize some specific motifs. Protein quantification uses gel-based methods including differential staining of gels with fluorescent dyes (difference gel electrophoresis). Gel-free methods include various tagging or chemical modification methods, such as isotope-coded affinity tags, metal-coded affinity tags, and combined fractional diagonal chromatography. Modern day gel electrophoresis techniques often leverages software-based image analysis tools to analyze biomarkers by quantifying individual "spots" on a scanned image of a two-dimensional electrophoresis (2-DE) product and calculating the separation between one or more proteins. Structural proteomics is concerned with the high-throughput determination of protein structures in three-dimensional space. The most common methods are X-ray crystallography and NMR spectroscopy.

Proteomic studies are considered an useful tool in many different fields related to aquaculture. For example, the study of the proteins involved in the different mussel larval stages and their comparison with other bivalve species that coexist in the same habitat can permit the isolation of specific proteins and help to develop immune methods of larval identification from plankton samples with the aim of predicting the patterns of mussel settlement. Proteomics can also facilitate the work of the growers (López et al., 2005; Lorenzo-Abalde et al., 2005). The first studies carried out in molluscs were designed to characterize differences in protein expression among phylogenetically related mussel species with different ecological requirements (López et al., 2002; López et al., 2005; López, 2007). These studies showed higher levels of expression in some stress-related proteins, such as HSP70 and calreticulin, in Mediterranean mussels, *M. galloprovincialis*, from intertidal areas compared with those animals cultured in rafts, which inhabit a more homogeneous environment. Nowadays, proteomic tools are widely used for different purposes. The protein expression signature technique has been successfully used to detect exposure to heavy metals and environmental stress. The risk of environmental toxins depends on the metabolic balance between bioactivation and detoxification enzymes. Therefore, the agents that can alter enzymatic expression levels are critical factors in toxicity.

The study of these changes as well as the quantitative analysis of the proteins involved in responses to contaminants is possible using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. This methodology, a MALDI-TOF variant, makes it possible to detect changes in protein translation with high efficiency. Jonsson et al. (2006) used polyclonal antibodies against cytochrome P450 to investigate responses in the blue mussel (*M. edulis*). Furthermore, a proteomic approach was used to elucidate exposure-related protein changes. The identities of isolated proteins were determined from peptide mass fingerprints obtained from MALDI-TOF MS analyses. Difference gel electrophoresis and MS liquid chromatography coupled with 2-DE techniques were also used in blue mussel to identify signatures associated with exposure to marine pollutants (Rodríguez-Ortega et al., 2003; Apraiz et al., 2006; Amelina et al., 2007). A combination of western blotting, proteomic, and epi-fluorescent microscopy techniques was used to examine the presence of shell matrix proteins in *C. virginica* (Johnstone et al., 2008). Ronzitti et al. (2008) used proteomic analysis to detect and identify biomarkers of biotoxin contamination in shellfish. Two-dimensional electrophoresis and mass spectrometry were used to identify markers of QX disease in Sydney rock oysters, *Saccostrea glomerata* (Simonian et al., 2009). In the clam, *R. decussatus*, the effects of cadmium on protein expression profiles were also analyzed using proteomic tools (Chora et al., 2009). The proteomic response of the mussels *M. galloprovincialis* and *M. trossulus* were studied using two-dimensional gel electrophoresis and tandem mass spectrometry (Tomanek and Zuzow, 2010).

Future Applications of Mollusc Genomics to Aquaculture

Genomic methods offer useful tools for studying the molecular basis of biological characteristics of great interest in aquaculture, such as disease resistance and stress. As Saavedra et al. (2009) stated, there are several problems affecting bivalves that can benefit from these genomics approaches.

One of the most important factors for aquaculture is the presence of toxins with a phytoplanktonic origin. These toxins can produce red tides (Bricelj and Shumway, 1998; Burgess and Shaw, 2001) that cause several diseases in humans after consumption. The accumulation and depuration rate of each toxin varies and the molecular and cellular bases of the response to phytoplanktonic toxins are not well known so far. Genomic tools will provide a perfect means of studying these processes.

Very little is known about the biology of the larval phases of bivalves, especially from a cellular and molecular point of view. Genomic techniques are perfectly suited for the study of larval stages that can be a problem in some bivalves cultured in hatcheries. Diet design and seed growth could be addressed using molecular techniques (Blankenship and Yayanos, 2005; Knauer and Southgate, 1999; Singh and Zouros, 1978). In hatcheries, the proper control of sex determination and sexual maturation are very important, but these processes are not well studied so far (Avise & et al., 2004; Guo et al., 1998). Molluscs do not exhibit chromosomal sex determination. Likewise, little is known about the molecular mechanisms involved in gonad differentiation and the maturation of the gametes, which are perfect subjects for genomic studies. In addition, growth is an ideal characteristic to be studied using genomic techniques, as well as aspects related to disease resistance or stress.

Acknowledgments

The work in our laboratory on mollusks genomics is supported by the Spanish Ministry of Science and Innovation (Project AGL2008-05111/ACU) and by the EU Projects REPROSEED (245119) and BIVALIFE (266157).

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Index

Functional Genomics in Aquaculture, First Edition. Edited by Marco Saroglia and Zhanjiang (John) Liu.