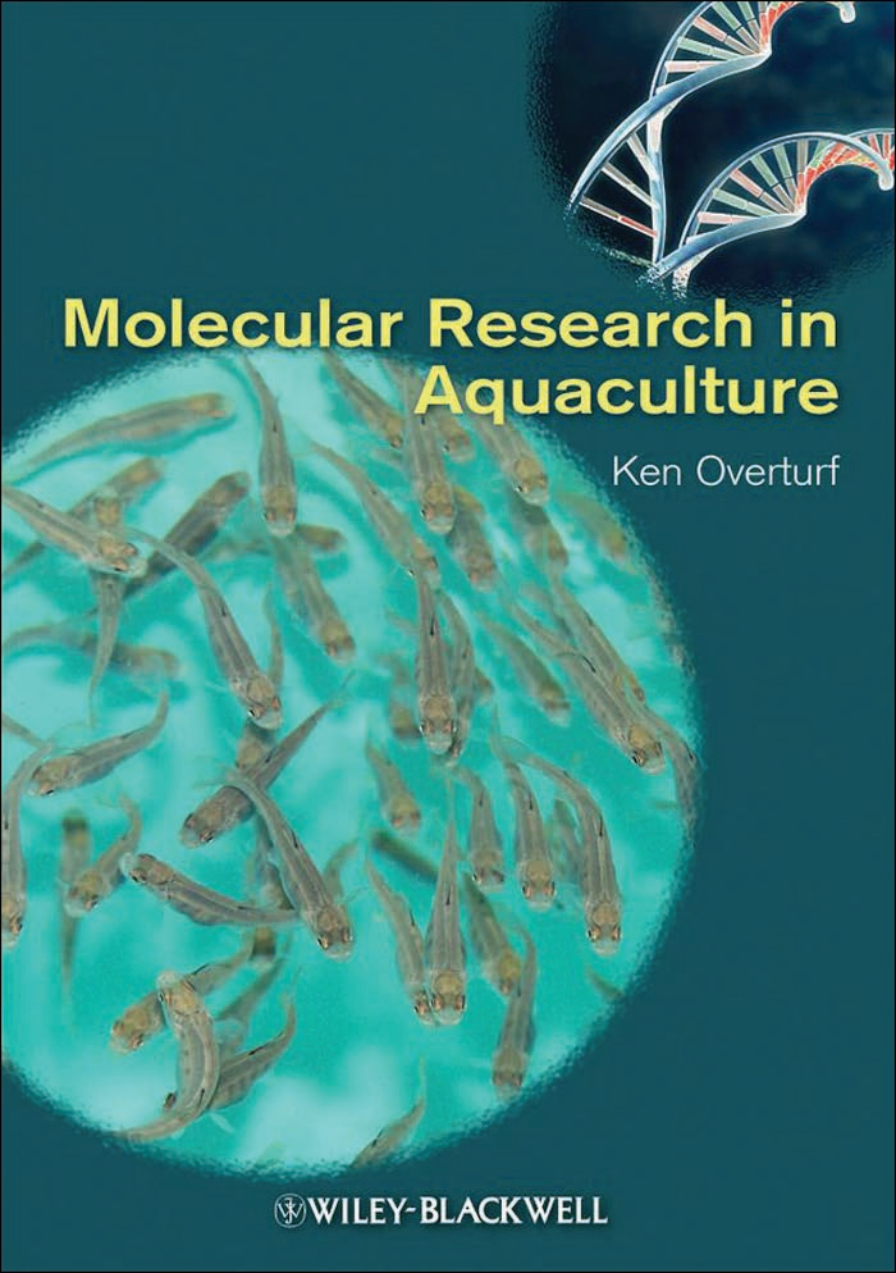

Molecular Research in Aquaculture

Ken Overturf

WILEY-BLACKWELL

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Preface

The aim of this book is to provide a basic understanding of the modern molecular techniques currently used in aquaculture research, primarily finfish aquaculture, such that readers can develop an understanding of both the power and limitations of molecular biology. Recently, in the scientific literature, there has been a profusion of information pertaining to genetics, genomics, transcriptomics, proteomics, and other related buzzwords meant to describe molecular research relating cellular events to physiological traits. The material covered in the chapters of this book provides background to newcomers interested in molecular techniques and a description of the current state of research and scientific understanding of gene regulation in regards to physiological areas important to aquaculture. For researchers currently working in the field on specific genes, pathways, or traits, this text provides invaluable information that relates molecular function to fish physiology.

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Chapter 1
Convergence of Aquaculture and Molecular Biology

Ken Overturf

Introduction

More than 1 billion people rely on fish as their main protein source. Of the world's food fish supply for consumption, more than 48% is supplied by aquaculture. Currently, more than 240 diverse species are produced by aquaculture; however, within aquaculture only 10 species constitute approximately 69% of the production, while 25 species account for approximately 90%. On a weight basis, finfish account for 85% of all aquaculture production (FAO 2005). Most farmed finfish are of the class Teleostei (teleosts), which contains 96% of all fish species, and are some of the most diverse, which is exhibited in their behavior, diet, reproduction, and habitat (Nelson 1994). Finfish also play a significant role in research and have become more prominent as a research subject during the past three decades.

The human population has continually developed methods for the increase of aquatic products for food production. However, the roots of aquaculture, as illustrated by the writings of Fan Li, go back to more than 4,000 years ago when the Asian emperors maintained stocks of their favorite fish in ponds. Until the late nineteenth century aquaculture research mainly consisted of domestication and rearing of wild stocks in captivity. Initially, rearing species harvested from the wild was the main form of aquaculture. Closing the life cycle of certain fish species allowed them to be maintained, grown, and spawned without having to constantly capture fish from the wild. As the production of fish products moved beyond the provisional rearing of fish for individuals and into product marketing, demand and economics of production led to intensification of aquaculture. Today, aquaculture occurs in multiple countries around the world and accounts for more than US$65 billion in trade. Also, when considering fish for human consumption and wild fish harvested for use in aquaculture diets, aquaculture is responsible for more than half of the world's fishery production (FAO 2005).

The scope of early aquaculture studies was greatly influenced by the production systems in use. Preliminary studies in fish culture began with the evaluation of intended improvements in the growing environment and the type of feed that would either reduce loss to disease, enhance growth, or facilitate ease in rearing the animals. These types of studies mainly depended on the species and the area in which rearing occurred and were usually performed by private entities to improve production for their specific case. Rearing typically occurred in ponds; this limited the chance of escape and also established boundaries for the rearing of freshwater fish species. The concept of cage culture arose next, whereby existing bodies of water could be utilized while still maintaining a safe and secure enclosure. For practical and economical reasons, most

of the original work was performed in freshwater while all marine fish used for food were harvested from the wild.

The course and development of aquaculture has obviously varied widely throughout the world, according to location, environment, and population. Historically, with the intensification of aquaculture began the initial stages of research, with individuals or groups attempting to qualitatively experiment to determine the optimal water conditions, stocking densities, and diets for the available species that performed best under local conditions. However, it was not until the early nineteenth century that aquaculture research could essentially be considered an applied science. Some scientific studies related to aquaculture were undertaken in government and academic laboratories prior to this period; however, they were relatively limited in number and scope (Stickney 1996). Nevertheless, progress was made through trial and error and through careful observation of cultured animals while attempting to re-create optimal natural conditions in captive environments.

As was typical for the time, most of the knowledge that was gained in early studies was never written and published in the research journals or the popular press. Information on the practices for the rearing and care of fish was passed down by traditional methods. In the late 1800s, state and federal government agencies had developed the technology for the production of millions of eggs and small fish for stocking into marine, coastal, and freshwaters. Yet, the technology to spawn some of the species mentioned in the literature has only recently been redeveloped (Stickney 1994). Still there are several species where little or no breeding of captive broodstock has occurred and all broodstock or fry are obtained from the wild. This is still a problem that is being researched for new species, especially when considering larval diets and rearing conditions.

The development of a vitamin-free purified diet that supported growth but allowed for the testing of the qualitative and quantitative requirements for vitamins was a landmark discovery by John Halver in the 1950s in aquaculture research on the production of diets for the maintenance and study of commercial and research species (Halver 1957). Before this discovery, diets needed to be supplemented with multiple nutrient factors to ensure animal health from fry stages to fully mature individuals. Modification of dietary formulations has been necessary to study amino and fatty acid requirements. With the development of complete diets, initial studies involving selection for improvement of performance, chromosomal manipulation, and sex reversal started. Dietary research has now expanded tremendously with ongoing studies, evaluating the nutritional requirements of several different species at all life stages, improving immune performance, developing diets composed of material from sustainable sources, and modifying formulations now specific for the development of newer species for aquaculture (Amar et al. 2000; Cahu and Infante 2001; Twibell et al. 2003).

Until recently, aquaculture was not a distinct scientific discipline. Rather, it was the application of discrete scientific disciplines such as nutrition, genetics, physiology, and health management to aquatic species. Culturists and researchers alike obtained information from studies involving other agricultural animals or scientific species and then integrated this information into traditional practices and research studies and applied it to aquaculture research. To some extent this practice is still followed today. However, with the growth and economic development of aquaculture has come increased funding, fueling the development of research programs and departments and even entire institutes devoted specifically to aquaculture research. The rapid

advancement of scientific research found today in aquaculture is due to the rapid application of technology, which is often adapted from human or medical research. As this technology becomes available for agriculture research and as the methodology becomes developed and refined, it is then incorporated into studies involving aquaculture species. Since 2000, the number of published scientific articles related to aquaculture research is fivefold greater than the number of articles published in the entire previous decade. And typically most of the articles now being published are more of a scientific nature, whereas some of the earlier literature related to fishery conditions and were not specifically dealing with research in aquaculture. Unlike traditional agriculture where a limited number of species are under study, in aquaculture more than 240 diverse species are being evaluated, cultured, and studied in multiple different environments. The diversity of aquaculture species defies a united front to the scientific advancement of studies for these species, as each is in a different phase of development. Also, the value of the product and its potential usefulness as either a research animal or a food animal impacts research funding as has been seen in recent years. However, the use of some fish species as models for the analysis of development and so forth has enhanced our general knowledge of fish anatomy and physiology. In terms of research, fish are the third most commonly used experimental animal after mice and rats in countries such as the UK (Ostrander 2000). This increase is a result of the rapid development of the aquaculture industry, regulatory requirements for testing involving fish as indicators of environmental change, and the use of fish as a replacement for mammals in biomedical, pharmacological, and genetic research. Several aquatic species such as zebrafish, fugu, rainbow trout, catfish, Atlantic salmon, tilapia, and bivalves are exclusively used in several scientific and agricultural research programs.

Of these species, the zebrafish is the most extensively studied fish species. Although not reared commercially as a food animal, zebrafish are nevertheless economically important, with specific stocks and species being reared and sold around the world. Studies with zebrafish began in the 1970s by Oregon scientist George Steisinger (Detrich et al. 1999). The identification and study of mutations in zebrafish has been extremely successful in providing an understanding of early embryonic development. Mutagenesis screens have provided proof of principle that classical forward genetics can be used to understand vertebrate development. In other vertebrate research species, embryogenesis is difficult to study as it occurs within the uterus. In the late 1970s, researchers began to use zebrafish as an organism for study since it was readily available, had a relatively short generation time, and produced large clutches of embryos, as well as since all its developmental stages could be easily visualized. Furthermore, the zebrafish embryo possessed a simple organization, containing fewer cells than other vertebrate species, and the embryo possessed transparent cells that are accessible for manipulative studies, which can be injected with tracer dyes to track emerging cell lineages. Molecular, cellular, and developmental studies of mutant zebrafish collections have yielded a wealth of knowledge regarding vertebrate development. Currently, there is zebrafish research on the genetics of behavior and the generation of conditional mutants contributing to the dissection of gene function. Other related important genomic research species are the fugu or pufferfish (*Tetraodon nigroviridis*) and medaka (*Oryzias latipes*).

Rainbow trout have been a popular species for research. More is known about the physiology and biology of rainbow trout than of any other aquatic species. Rainbow

trout excel as a physiological and genetic model organism. The fact that they are large in size allows for isolation and harvesting of large amounts of specific tissues and cell types for biochemical, immunological, and molecular biological analyses. Much is known about rainbow trout from research involving their natural populations, established clonal and transgenic lines, and extensive generated sequence information (Thorgaard et al. 2002). Furthermore, regarding salmonid genomics research, several countries (including Norway and Canada) have generated salmonid libraries and microarrays, mainly geared toward Atlantic salmon, for research use. Currently, a wealth of information is being generated for a number of other species, including cod, flounder, and tilapia.

In early studies using fish, as early as 1863, a majority of experiments involved fish as monitors for toxicity in aquatic environments or as a readily available source of tissue (Hunn 1989). During the middle of the last century, toxicity tests with fish increased, mainly due to concerns regarding the widespread use of pesticides. Since then, fish have remained as a standard for use in bioassays for acute toxicity monitoring and in the evaluation of chemical toxicity. With increasing concerns regarding industrial contamination, fish are actively used as environmental biomarkers in monitoring the environmental status of both fresh and marine water systems (Rand and Petrocelli 1985). By the seventeenth century, biologists throughout Europe were beginning to refine and expand their scientific observations. In the late 1600s, Anton van Leeuwenhoek used microscope to observe and describe sperm from fish and other organisms. In 1668, the court physician Francesco Redi used the fish and other animal tissue to refute the idea that flies could develop spontaneously from putrefying flesh. And then in 1686 in England, Francis Willughby published a manuscript of detailed fish drawings sufficient for the identification of several species. The studies by Borelli on the mechanics of muscular action and swimming action led to models on how animals moved and how fish swam (Ostrander 2000).

Nigrelli (1953) in an overview on the "utilization of fish in biological research" offers a chronological synopsis of fish as experimental animals. In this overview, Nigrelli asserts that at one time most important researchers have probably used fish in their research. The fish mummichog (*Fundulus heteroclitus*) was used in a significant number of studies dated to the late 1900s by researchers at Woods Hole, Maine. Many experiments were conducted on mummichog genetics, pigmentation, and endocrinology. Perhaps one of the earliest known species worked with was *Carassius auratus*, or the common goldfish. Because of their size, availability, and early domestication, tropical fish were a favored species for researchers. Early research dealt with egg or gross anatomical development, physiology, viability under different environmental conditions, and the effects of chemicals on development. Beginning in the 1970s and extending to the present day, the concept of fish as experimental models has taken hold, and they have proven to be an indispensable asset to research advancement.

Until recently, in most scientific studies involving fish or their eggs, fish were used as components for monitoring an effect, typically of waterborne toxins, and the studies were not specifically fish related. However, with the burgeoning economic importance of aquaculture and development of fish models, some aquatic specimens have come to the forefront of modern research, and this information is now being used to improve aquaculture.

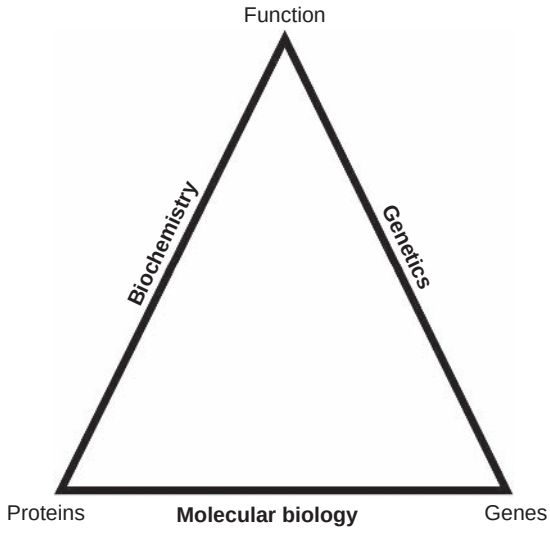

Figure 1.1. Interrelationship between scientific disciplines and studies on how genes and proteins function.

Molecular Technology

Although fish farming and aquaculture research has been practiced for several thousand years, the advent of research on the molecular level is a more recent occurrence. The history of molecular biology began around 1930 with the convergence of various previously distinct biological disciplines including biochemistry, genetics, and virology (Figure 1.1). However, the basics of molecular research was provided even earlier with studies in chemistry, physics, and microbiology. Researchers in these areas began integrating their research with the hope of obtaining an understanding of life at its most basic level. The combination of research goals in these areas of science led to what is now known as molecular biology. In an article in the journal *Nature*, Astbury (1961) once described molecular biology as not so much a technique as an approach for studying physical manifestations in form, and from this determining their development from biological molecules and their function (Bernal 1963). James Crick in 1957 coined the term "the Central Dogma," which he used to describe the biological flow of sequence information from nucleic acids, including DNA replication, RNA transcription, and the translation of proteins and their processing. Basically, this premise translates into a working description for the replication of cellular DNA, transcription of DNA to RNA, translation of RNA to protein, and the action of translated proteins on cellular and physiological levels relating to development and whole-body traits. The term "molecular biology" most likely originated in 1938, when Warren Weaver used it to explain the working of particles involved with life. These particles, we now know, consist of nucleic acids and proteins. In its modern sense, molecular biology attempts to explain the phenomena of life, starting with the biological components that contain the information necessary to replicate and give rise to organisms. The current definition of molecular research involves studies involving DNA, RNA, and protein,

as well as research involving their tissue-specific levels, activities, pathway linkages, function, and related involvements with physiological changes seen in an organism. A brief chronological description of the historical events that occurred involving nucleic acids, proteins, and genetics provides a better understanding of the current status of molecular research.

The characterization of chemical molecules that determine the physiological makeup of living organisms gained momentum with the birth of physiological chemistry in the nineteenth century and biochemistry at the beginning of the twentieth century. When the molecular revolution is evaluated within the context of biological history, it is easy to note that it is the culmination of a long process that began with the first observations through a microscope in the eighteenth century. The aim of early biologists was to deduce the functions of living organisms by describing their organization at the microscopic level, while chemists were obviously more interested in studying the chemical compounds found in organisms. Between the molecules that make up chemical compounds studied by chemists and the tiny structures visible under the optical microscope, such as the cellular nucleus or the chromosomes, there was an obscure zone, "the world of the neglected dimensions," as it was called by the chemist Wolfgang Ostwald. This zone was populated by colloids, biopolymers, and chemical compounds whose structures and properties were not well defined. Most of the important early scientific advancements involved working with materials that were too small to visualize with a microscope yet possessed the ability to transfer information from parents to offspring in a mysterious manner. This line of research required the generation of techniques for the indirect study of the experimental samples. Eventually, through the culmination of related information gained from biochemistry, genetics, and physics, our current understanding of the molecular components controlling inheritance, development, and physical traits was pieced together.

Nucleic acids were first isolated in 1869 by Friedrich Miescher, when he discovered a weak acid in white blood cells that he referred to as "nuclein." Miescher isolated a pure sample of this substance from salmon sperm, and in 1889 Richard Altmann, a student of Miescher, termed the isolate "nucleic acid." At that time, this substance was found to exist only in the chromosomes of cell nuclei, and biochemists initially isolated DNA and RNA concurrently from cell nuclei. Researchers soon discovered that the nucleic acids isolated had a polymorphic nature and it was later realized that there were two distinct types: RNA which contains ribose and DNA which contains deoxyribose.

In 1929, Phoebus Levene at the Rockefeller Institute identified the basic components of DNA, which consisted of four bases, sugar, and a phosphate chain, and then determined how they were linked. He called each of these units a nucleotide and suggested that a molecule of DNA was composed of a string of nucleotide units attached together by phosphate groups. These phosphate groups provided the structural support for the molecule. However, Levene thought that the chain was short and that the bases were repeated in the same fixed order. In 1937, William Astbury produced the first X-ray diffraction patterns from DNA. He was not able to propose the correct structure, but the patterns showed that it was regular and repetitious, suggesting that it might be possible to deduce the structure.

The structure of DNA was finally elucidated in the 1950s, when three groups made it their goal to determine its cellular assembly. By using different methods, Maurice Wilkins and Rosalind Franklin at King's College London, Linus Pauling at Caltech, and Francis Crick and James Watson at Cambridge attempted to generate

an accurate structure of the DNA molecule. Piecing together the information from all three groups, which included the discovery of helical shapes in proteins by Pauling and the X-ray diffraction information from Wilkins, Watson and Crick attempted to build a physical model of the helical structure using the chemical structure of the nucleotides and their linkages. A final crucial piece of information came from the work of Erwin Chargaff, who had reported that although the proportion of the four nucleotides varied among different DNA samples, the proportion of pairs of the nucleotides were always the same. Restricting themselves to the development of a model that they considered as chemically and biologically reasonable, Watson and Crick in 1953 developed the first accurate model of the molecular structure of DNA (Figure 1.2).

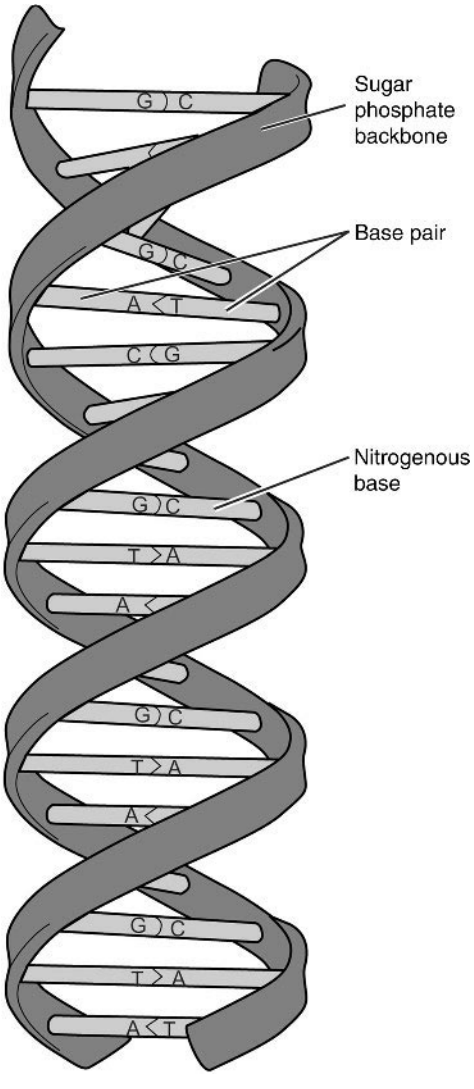

Figure 1.2. Diagram of the helical form of DNA, detailing the position of certain chemical components.

In 1962, Watson, Crick, and Wilkins jointly received the Nobel Prize for determining the structure of DNA.

Experiments by Meselson and Stahl in 1958 proved that DNA was semiconservatively replicated. This information helped to further confirm the double-helical model proposed by Watson and Crick, who later showed that the genetic code consisted of triplicate bases, termed codons. Later, Har Gobind Khorana interpreted the genetic code and its function in protein synthesis. In 1964, Howard Temin demonstrated, by using RNA viruses, that the direction of DNA to RNA transcription could be reversed.

Molecular Biology and Genetics

Prior to the successful characterization of the structure of DNA with new technologies developed by chemists and physicists, such as X-ray diffraction, electron microscopy, ultracentrifugation, and electrophoresis, parallel research was being performed in genetics by studying how genetic traits were physically transferred from parents to offspring. These studies went beyond evaluating the structure and function of the macromolecules. Scientists were attempting to link the action of unknown biological compounds with biological function. In 1865, Gregory Mendel published "Experiments in Plant Hybridization." Through the careful crossbreeding of pea plants, Mendel was able to determine how specific traits were passed from generation to generation. During the late 1800s Walter Flemming and coworkers showed that chromosomes divide and are distributed equally during cell division, and in 1903 Walter Sutton hypothesized that since chromosomes appear to segregate in Mendelian fashion, they might function as hereditary units.

After the rediscovery of the work of Mendel through the studies of Hugo de Vries, Carl Correns, and Erich von Tschermack in 1900, the study of inheritance and how it was passed on moved forward rapidly with the work of Thomas Hunt Morgan, who in 1910 used the fruit fly, *Drosophila*, as a model organism for genetic studies. Morgan showed that genes are localized on chromosomes. Following this discovery, he continued working with *Drosophila* and, along with numerous other research groups, confirmed the importance of genes in the development and physiology of organisms. On the basis of the work of Morgan and his own research, Alfred Sturtevant in 1913 was able to produce the first genetic map of a chromosome and demonstrate the linear arrangement of genes. It was not until 1931, however, that Jean Brachet demonstrated that chromosomes were the cellular components that contained DNA and that RNA was present in the cytoplasm of all living cells. Despite these discoveries, the chemical nature of genes, their structures, and their mechanisms of action remained elusive. Researchers from multiple disciplines committed themselves to determining the structure and elucidating the complex relations between genes and proteins.

Max Delbrück, Nikolai Timofeeff-Ressovsky, and Karl Zimmer published results in 1935 suggesting that chromosomes were very large molecules whose structures could be changed by exposure to X-rays and that by so altering their structure it was possible to change the heritable characteristics governed by those chromosomes. In 1928, Frederick Griffith demonstrated the potential for nonpathogenic bacteria to acquire traits from dead pathogenic bacteria when cocultured in mice. Unfortunately, Griffith was killed at work during an air raid. However, in 1943 Oswald Theodore Avery and a team of scientists were able to duplicate some of Griffith's research results and discovered that traits associated with one form of the bacteria pneumococcus could be

transferred to another form of the same bacteria merely by making biological material from a killed form available to living bacteria. Then, quite unexpectedly, it was found that these transferred traits were heritable. Avery identified DNA, and not protein, as the material responsible for the transformed bacteria and called the transfer of traits the transforming principle.

Also during the early 1940s, George Beadle and Edward Tatum were able to demonstrate the existence of a relationship between coded genes and expressed proteins within an organism. Beadle and Tatum switched from using *Drosophila* as their genetic animal model to a more appropriate model organism, the fungus *Neurospora*. By constructing mutant strains that required specific amino acids or vitamins, they verified, by means of gene mutations, that individual genes were responsible for specific steps in the metabolism and synthesis of vital nutrients. The culmination of this work in 1941 led to the proposal of the "one gene–one enzyme hypothesis," in which the concept is that a single gene specifies a single enzyme or protein rather than a complex set of characteristics. In 1944, Oswald Avery, working alongside Alfred Mirsky at the Rockefeller Institute of New York, demonstrated that genes were composed of DNA. In 1952, Alfred Hershey and Martha Chase, in what is now termed the Hershey–Chase experiment, confirmed that the genetic material of the T2 bacteriophage, a virus that infects bacteria, was made up of DNA. In 1961, Francois Jacob and Jacques Monod demonstrated how certain specific proteins, called regulative proteins, latch onto DNA at the edges of the genes and control the transcription of these genes into messenger RNA. A milestone during the process of deciphering the link between DNA and protein was provided by the work of Linus Pauling who for the first time linked a specific genetic mutation in patients with sickle cell disease to a demonstrated change in an individual protein, the hemoglobin in the erythrocytes of heterozygous or homozygous individuals.

Between 1961 and 1965, researchers were able to determine the relationship between the information contained in DNA and the structure of protein. They found that the nucleotide arrangement of DNA on chromosomes provides a "genetic code" which is followed in order to make a complementary sequence of the nucleic acid RNA. This code then corresponds to a chain of amino acids that are linked together by ribosomes during translation of the RNA sequence to generate a protein.

Thus, several of the key discoveries of molecular biology took place in a period of only about 25 years. Over the next 20 years, new and more sophisticated technologies allowed for the isolation and characterization of genes and their function. This effort continues today.

Links between Molecular Biology and Genetics and Biochemistry

Biochemistry and genetics also had significant impacts on the development of molecular biology. Although a large amount of research had already been done in regard to protein chemistry since the late 1700s, during the first half of the twentieth century significant progress was made in our understanding of the role of proteins in metabolism and even in genetics. Prior to the 1900s biologists and chemists studied fermentation, the liquefaction of meat when exposed to stomach secretions, and the conversion of starch to sugars, but had yet been unable to determine the mechanisms causing these changes, the link being each of these processes is catalyzed by a specific enzyme. As a consequence, the study of proteins, their structures, and syntheses became one of the principal objectives of biochemists.

Protein Biochemistry

In the late 1830s, the Dutch chemist Gerhardus Johannes Mulder began elemental analyses of common animal and plant proteins. Unexpectedly, he discovered that all proteins had nearly the same empirical formula. Mulder's professor Jons Jakob Berzelius proposed the term "protein" for these isolated substances. Mulder went on to identify amino acids as degradation products of proteins and even determined the correct molecular weight of several amino acids. Mulder's analysis of a pure isolated product suggested a weight that was much greater than that for other known molecules under study. Working against skepticism of the scientific community that such long macromolecules would be stable in solution, in 1902 Franz Hofmeister and Emil Fischer concurrently proposed the idea that proteins were linear polymers of amino acids linked by peptide bonds. It was not until 1920 that Theodor Svedberg was finally able to demonstrate, by using analytical ultracentrifugation, that proteins were macromolecules of well-defined composition. Later within the same decade, James Sumner was able to demonstrate, by using the enzyme urease, that proteins are not merely carriers but are responsible for enzymatic function. Sumner's method to isolate and crystallize proteins was extremely important because it eventually proved essential to determining their structures by X-ray crystallography. Early research with proteins was extremely difficult because most proteins were difficult to purify in more than milligram quantities, even using the most modern methods. Hence, early studies focused on proteins that could be purified in the largest quantities available, such as those found in blood, egg whites, and digestive/metabolic enzymes obtained from slaughterhouses. Several techniques of protein purification were developed by Edwin Joseph Cohn during World War II in an attempt to purify blood proteins for use in treating wounded soldiers. Then in an exceptionally alturistic gesture during the late 1950s, the Armour Hot Dog Co. purified 1 kg (=1 million milligrams) of pure bovine pancreatic ribonuclease A (RNase A) and made it freely available in 10-mg batches to scientists around the world. This generous act made RNase A the model system for protein basic research for the next several decades.

Studies in protein formation and folding began around 1910, when Henrietta Chick and C.J. Martin showed that the flocculation of a protein was composed of two distinct processes: First, during denaturation the protein becomes less soluble, enzymatically inactive, and more chemically active; then, the protein begins to precipitate from solution. In 1929, Tim Anson and Alfred Mirsky proposed in a paper that denaturation was a reversible process, a hypothesis that was widely ridiculed at the time. Anson later published an article with Linus Pauling detailing the energy states of proteins and suggested that denaturation was an all or none process in which the same changes occur that were documented by Chick and Martin. Around this time, Hsien Wu hypothesized that denaturation involved changes in the folded state of the protein, a purely conformational change that resulted in the exposure of amino acid side chains to solvents. According to Wu, exposure of side chains to solvent rendered the protein less soluble and more reactive, whereas the change in conformation was the reason for loss of enzymatic activity. In the early 1960s, Chris Anfinsen developed what he called his "thermodynamic hypothesis" of protein folding to explain the native conformation of amino acid structures. He theorized that the native or natural conformation occurs because this particular shape is thermodynamically the most stable in the intracellular environment. Anfinsen demonstrated that the three-dimensional state of the enzyme

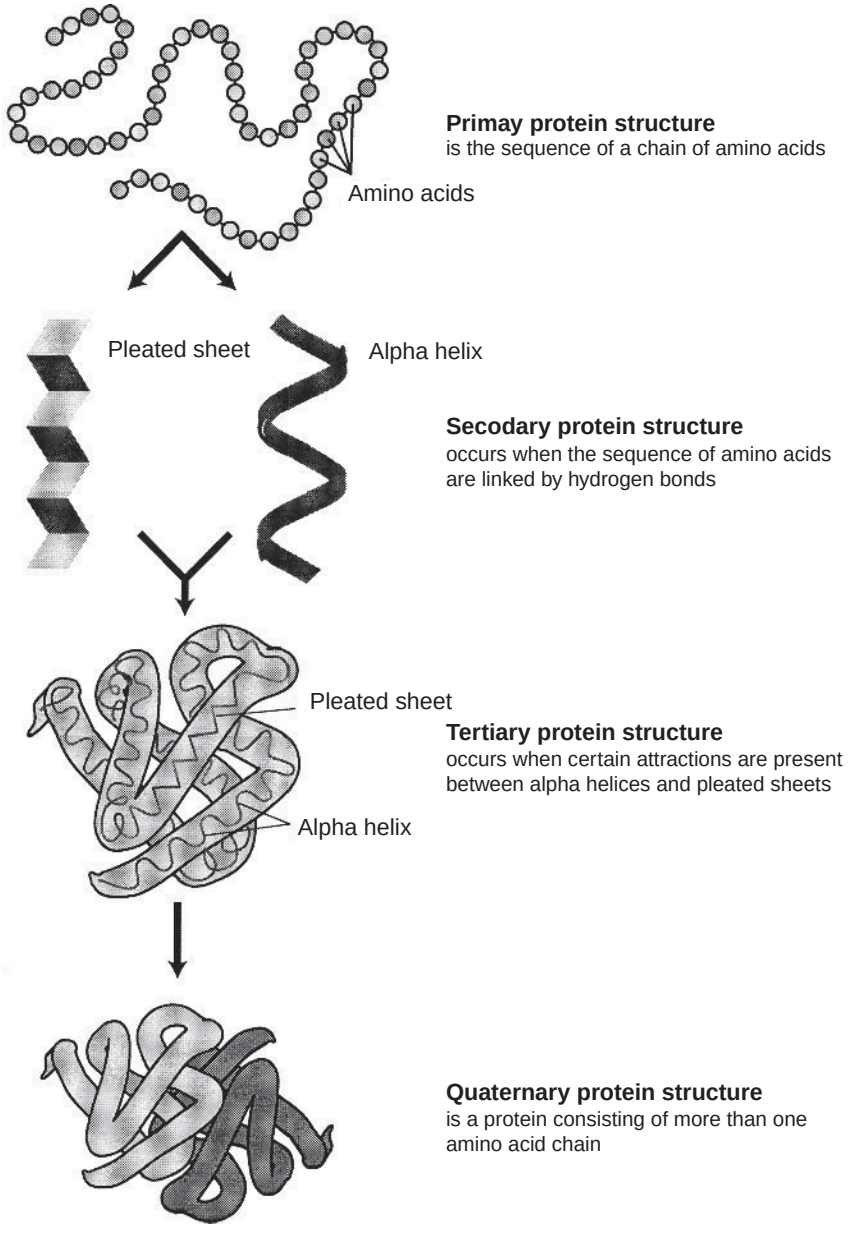

Figure 1.3. Protein folding structures. Examples of four different types of protein structures and their relative complexities.

RNase A was fully reversible with no external cofactors needed, verifying that the folded state represents the lowest free energy for a protein.

Linus Pauling was the first to correctly determine the secondary structure forms of the alpha helix and beta sheet of proteins (Figure 1.3). The stability of hydrophobic interaction for maintaining protein stability was first proposed in the late 1920s but

refuted until publication of an article on denaturation in 1959 by Walter Kauzman, based partly on work by Kaj Linderstrom-Lang. Arne Tiselius and associates were the first to demonstrate the ionic nature of proteins; however, Kaj Urik Linderstrom-Lang established that these charged bonds were accessible to solvent and not stringently bonded to each other.

The secondary and low-resolution tertiary structure of globular protein was investigated initially by hydrodynamic methods such as analytical ultracentrifugation and flow birefringence. The primary structure of protein was an extremely active area of research, when in 1949 Fred Sanger developed sequencing techniques for proteins and was able to sequence insulin. However, by the 1960s the first atomic-resolution structures of proteins were determined by X-ray crystallography and further clarified by the NMR method in the 1980s. As of 2009, the Protein Data Bank has over 55,000 atomic-resolution structures of proteins. Currently, cryoelectron microscopy of large macromolecular assemblies and computational protein structure prediction of small protein domains are two methods used that approach atomic resolution.

Research from these different scientific disciplines culminated in the 1970s and 1980s, when DNA sequencing allowed for (a) the separation and identification of different gene sequences along with the isolation and understanding of restriction enzymes for site-specific cleavage of DNA, (b) the development of cloning vectors for cloning and amplification of isolated sequences, and finally (c) the development of polymerase chain reaction for rapid amplification of nucleic acids. These and other techniques have opened up vast and remarkable techniques for determining physiological differences or changes that either are undetectable or cannot be discreetly measured by physical determination. In this book several of the methods or techniques most used or that are coming into common practice in current aquaculture research are discussed along with their practical applications to aquaculture.

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Chapter 2
Basic Molecular Laboratory Methods

Ken Overturf

Introduction

Molecular research is a mainstay in most research laboratories and is rapidly becoming an integral component in most aquaculture studies. Although the research projects and interests can be quite diverse between laboratories, there are several techniques that are now common practices such as sample isolation, polymerase chain reaction (PCR), cloning, and sequencing. In today's laboratories, it is common for researchers to have an interest in a specific gene, protein, or pathway. Depending on the organism the researcher is studying, the genes of interest may not have been previously cloned and sequenced. It would then be up to the investigator to clone and sequence the gene at either the mRNA or the genomic level. From this point there are many applications a researcher can follow to determine what role or at what level this gene, or more typically the protein, plays in the researcher's area of interest. Although by no means an exhaustive representation of all molecular techniques, this chapter outlines several of the methods that are commonly used in molecular research laboratories for isolating and evaluating nucleic acids and proteins purified from tissue. This chapter briefly touches upon the different techniques from initial isolation and crude preparation of samples to methods designed to dissect out the constituent components of the sample and study their function at the molecular level.

DNA, RNA, and Protein Extraction

The first step in any molecular protocol requires the isolation of a substance of choice. Typically, a high-quality, pure isolated sample is desirable, but this is not always the rule. The downstream application of the sample will determine the level of purification needed; typically, the higher the quality of the extract, the less contaminants it will contain. The first step of isolation usually consists of disruption of the cell membranes, either through the use of a reagent or by mechanical homogenization, and then releasing the cellular contents into a protective medium. The goal is to then separate the biological component of interest from the rest of the cellular material to a desired level of purity.

RNA Isolation

In order to limit degradation and potential harm from RNases, isolation of tissue for RNA extraction requires relatively quick storage upon sample harvest. There are several commercially available reagents such as RNAlater (Applied Biosystems, Foster

City, CA) that allows the researcher to harvest the tissue into collection tubes at room temperature and then isolate the RNA up to a month or more later, depending on storage conditions. Dissected tissue or collected cells are simply dropped into solution at room temperature. The solution permeates the cells and stabilizes the RNA. Other methods are to quick-freeze the tissue in liquid nitrogen, whereby it can then be cryopreserved at −80°C or below for up to 4 months. Otherwise one may directly isolate the material into an isolation medium such as a guanidium thiocyanate solution like TRIZol (Invitrogen, Rockville, MD) and begin isolating the RNA according to the protocol requirements.

Once the tissue is ready for RNA isolation, there are a multitude of kits that utilize either organic extraction and phase separation or affinity chromatography (i.e., spin columns). Spin columns have the ability to isolate total RNA or messenger RNA and function by preferentially attaching the RNA to a substrate and the extraneous material is washed away. The purified RNA is then released from the substrate in a final elution step. Typically with organic extraction, lysed cell components that are hydrophobic, such as membrane lipids and polysaccharides, will become trapped in the solvent and degraded, and the RNA (or DNA) can be extracted by decanting off the aqueous phase. The RNA can then be precipitated and washed by centrifugation in a 70% ethanol solution usually containing NH_4OAc. In most cases, a DNase step is necessary to remove any contaminating DNA material, and then the RNA sample should be resuspended in a molecular grade RNase-free solution, usually deionized water or a dilute buffer, at an appropriate concentration. If a cleaner sample is necessary, then further steps such as reisolating the sample by spinning through a resin column can be used for sample purification.

DNA Isolation

There are a great number of DNA isolation techniques and the method of preference can vary depending on the quality and quantity of DNA needed and the type of tissue from which extraction occurs. For researchers who are isolating DNA for genotyping, a simple protease K treatment for digesting the protein followed by precipitation in a salt solution and ethanol wash are sufficient. Some laboratories are even simply adding small amounts of sample tissue and protease K directly to PCR reactions and adding a preamplification step that will release enough material for PCR amplification of the desired amplified fragment. Other methods use detergents for disruption of cells and cell membranes to release the DNA, which is then isolated by centrifugation and pelletization of cellular material. Alternatively, the DNA can be isolated from the cellular material by running the mixed solution over a column that binds the DNA while the cellular material is washed through, after which the DNA is eluted in an appropriate solution. This is the main choice for isolation of plasmids from bacteria. Organic extraction is an alternative method for isolating DNA, especially from hard-to-lyse tissues or cells (Csaikl et al. 1998). Another method of cellular disruption that can be used for isolation of internal cellular components consists of using a homogenizer to shred the cells or through the use of small dense balls (glass or metal) added to the tubes, which are then vigorously shake the tubes in a mixer. However, it should be noted that these methods have a tendency to shear high-molecular-weight DNA. Some columns are available for the specific isolation of high-molecular-weight

DNA, or it can also be isolated by gradient centrifugation in a solution such as cesium chloride (Gross-Bellard et al. 2005). Modifications of these methods may also be used for the isolation of specific amplified or restriction-digested fragments isolated from agarose gels.

Protein Isolation

Protein isolation is somewhat different from the isolation of nucleic acids, depending on the level of purity required and the type of protein being isolated, additional steps may be required (Figure 2.1). Proteins are found contained in the cell wall, within the cytoplasm, and in the nucleus. These may also be found as individual proteins, homo- or heteromultimeric, protein–protein, and DNA–protein complexes. Typically, proteins are isolated into cellular fractions and not as a single protein species. This is because most procedures require a protein sample that contains little of other proteins or contaminants, which will interfere with the experiments for which this protein is

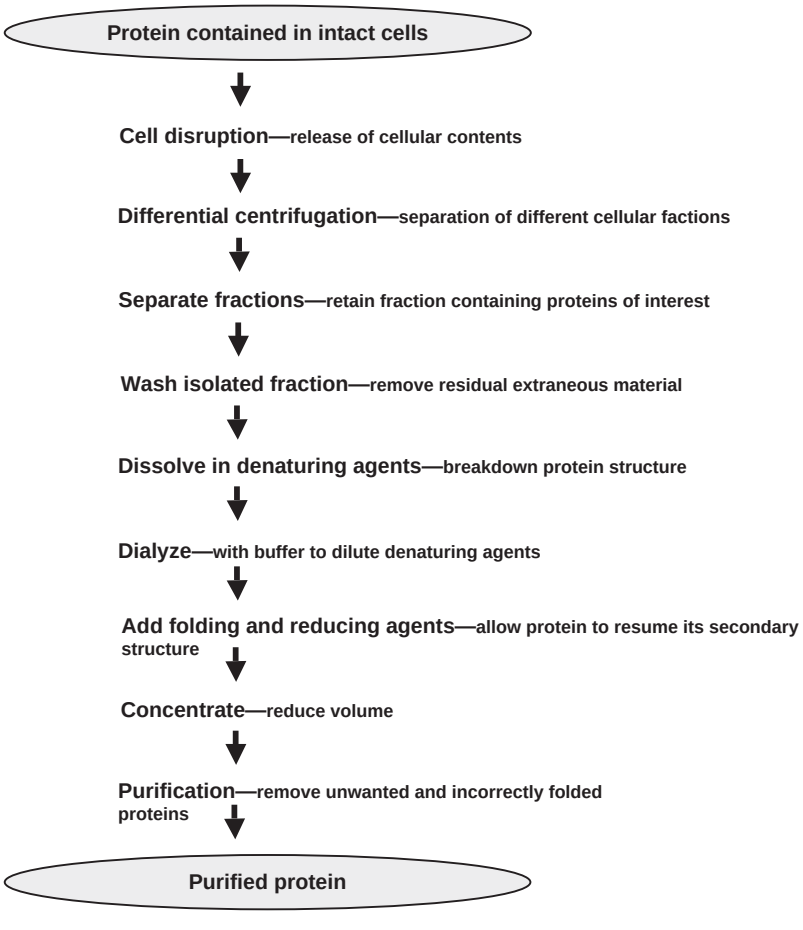

Figure 2.1. The potential steps that are involved in protein purification.

intended. Furthermore, isolation procedures sometimes need to take into account whether the protein structure and/or activity needs to be maintained in subsequent analysis.

Purification protocols are commonly divided into three stages. Stage 1 deals with cellular disruption and handling of the crude mixture of proteins and other cellular material present in the raw material; stage 2 involves secondary processing for the generation of a nearly homogeneous product; and stage 3 is considered a polishing step where minor contaminants are removed. These steps will vary according to whether the protein of interest is soluble, insoluble, or membrane bound.

If a single pure product is needed, for amino acid sequencing as an example, after further characterization, the protein can be isolated through the use of an antibody specific for an epitope on this protein, or by high-performance liquid chromatography (HPLC) column exclusion. For a protein without subunits or a protein with identical subunits, detection of a single protein band after one-dimensional (1D) gel electrophoresis under denaturing conditions or a single spot after 2D gel electrophoresis indicates a pure protein. If the protein consists of multiple subunits of different molecular sizes, purity is confirmed by detecting a single, stainable band after gel electrophoresis under nondenaturing conditions. Once the protein is demonstrated to be pure, an estimate of the molecular size of the protein may be made by comparing the elution volume of the protein from a conventional gel-filtration or HPLC size-exclusion column to the elution volumes of standard proteins. Almost all proteins can be isolated by a combination of conventional chromatography, HPLC, and electrophoresis.

Quantification of Nucleic Acids and Proteins

There are several methods for determining the concentration of nucleic acids. The methods used most involve quantification by spectrophotometry or fluorescence. However, there are some commercial products available that utilize blotting and relative color intensity to distinguish quantity (Kamiya Biomedical Company, Seattle, WA). Because nucleic acids absorb light at a peak wavelength of 260 nm, the concentration of DNA and RNA in solution is often calculated by using spectrophotometers. An optical density of 1 corresponds to approximately 50 ng/μL of DNA, 40 ng/μL of RNA, and 33 ng/μL of short single-stranded oligonucleotide. Often an A260/280 figure will be given to assess the purity of a sample in regards to protein contamination. Although this is still commonly used, this method, while good at determining levels of nucleic acid contamination in protein samples, is not very sensitive for the detection of contaminating protein in nucleic acid samples since the extinction coefficient is so much higher at 260 and 280 nm for nucleic acids when compared to protein. Another potential problem with reading samples at 260 nm on the spectrophotometer is that some contaminants such as phenol also absorb at 260 nm and can affect the detected concentration level.

For greater sensitivity and specificity, nucleic acids can also be quantified using a fluorescent intercalating dye. Ribogreen (Invitrogen, Carlsbad, CA), which is specific for RNA, and other similar reagents are intercalating fluorescent dyes that are added to a sample and used to determine concentration by measuring on a fluorometer. These reagents provide a broad linear range, are specific and highly sensitive, and consume

relatively small quantities of sample. For this method, a standard curve needs to be generated from which concentrations of unknowns can then be determined. Another method involves injection of the sample into microfluidic chips and reading them with an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA). This system allows for the separation of DNA, RNA, and protein, and can analyze both quantity and purity in as little as 1 μL of sample. This format also uses intercalating dyes, which are quantified after laser excitation.

There are several commercial kits available for the quantification of protein. Most of these assays are based on the absorbance of the protein bound to a dye. The Bradford method is a colorimetric assay that is based on a shift in absorbance of Coomassie dye when bound to protein. The protein samples mixed with dye are read at 595 nm, and an increase in absorbance at this wavelength is proportional to the amount of protein in the sample. This assay is linear over the range from 2 to 120 μg/mL of protein. It should be noted that the amino acid composition of the protein can affect the concentration since this assay mainly depends on the level of hydrophobic acids found in the protein. Modified versions of this assay are available using different forms of Coomassie dye that enhance the range of detection. The Lowry method relies on the use of copper in an alkaline solution that reacts with a phenol reagent turning blue. This assay is read at 750 nm, and the amino acid concentration and presence of acids can also influence the detected concentration of the sample. Most commercially available assays use some modification of these assays. Protein concentration can also be determined spectrophotometrically by reading at 280 nm. However, the extinction coefficient of proteins varies depending on both the amino acid sequence and the folded structure. A calculated estimation of a protein's extinction coefficient based on the amino acid sequence can be obtained using one of several online resources such as the Scripps protein calculator (http://www.scripps.edu/~cdputnam/protcalc.html). As previously discussed, contamination with DNA is more pronounced with this method. Most commercial assays provide bovine serum albumin as a standard, and protein concentrations are determined from plotting against a standard curve.

Sequencing

In molecular research, sequencing is used to determine the arrangement of bases, in the case of DNA, or amino acids for protein. The information obtained can assist in identifying and characterizing the function of an isolated sample. This has recently become a rapidly advancing technical area of molecular biology; where, only recently, most laboratories used radioactivity labeling, ran samples on polyacrylamide gels, and then analyzed the autoradiographs. The current techniques show significant advantages. This section briefly describes the basics of the method and then mainly deals with how sequencing is currently performed in most modern laboratories.

DNA Sequencing

In regards to standard methods for DNA sequencing, the chain termination method developed by Fred Sanger and coworkers in 1975 was the start of the modern era of sequencing. Although other methods such as the Maxam and Gilbert chemical

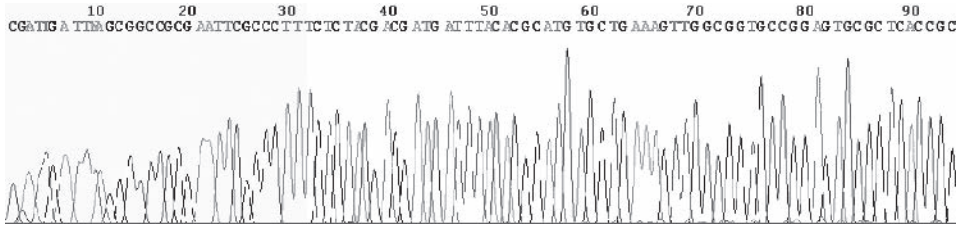

Figure 2.2. A sequencing electropherogram.

sequencing have been developed and used, most sequencing performed in laboratories today is done using the chain termination method. Initially for this method, complementary labeled primers were extended by polymerases, and the reactions were stopped by the incorporation of dideoxy nucleotides. Alternatively, the dideoxy termination has been modified into what is known as dye-terminating sequencing. In this technique, the fragments are size separated by capillary electrophoresis and the base determination of each fragment is determined by optical detection and the information processed directly by an attached computer, which will display the sequence information by showing corresponding fluorescent peaks as an electropherogram (Figure 2.2). Sequencing is typically performed in a plate format with samples being run in 96 or 384 well plates and with the use of automated sequences and plate stackers, sequencing can be run continuously.

Another technique termed *Pyrosequencing* is being commonly used in genome sequencing, to establish evolutionary relationships between organisms, to establish patterns between similar closely related groups, to evaluate mixed populations, and to study genetic similarities in responses between groups such as for predisposition to disease. This is the first commercially available large-scale format with the ability to sequence up to 100 Mb in as little as 7 hours using the high-throughput method licensed by 454 Life Sciences in Branford, CT. In this technique, the DNA to be sequenced is broken up into smaller strands, typically less than 1,000 bp, denatured to form single-stranded DNA and attached to microscopic beads or possibly a fixed surface. Then, PCR is performed on each strand and with the addition of each base a chemiluminescent signal is produced that is specific for each of the four bases; this signal is detected and recorded by a camera and the attached computer then analyzes the received information and outputs the sequence. Other similar high-throughput methods are currently being developed and coming online, and this along with other aspects regarding sequencing is further discussed in Chapters 5 and 12. After a sequence is known, that sequence can then be aligned against a number of sequences in databases such as NCBI genbank, which can be found on the internet at http://www.ncbi.nlm.nih.gov/Genbank/index.html or the following web site for The Institute for Genomic Research (TIGR) (http://www.tigr.org/). This information can then be used to identify the gene for which that sequence coded and potentially important areas of the gene involved with its regulation and function.

Protein Sequencing

Protein sequencing involves determining the succession of amino acids that constitute peptides. Mass spectrometry is now the primary method used for sequencing most

proteins, but for difficult proteins and for some smaller scale applications the Edman degradation method is still used. Automated sequences are available that utilize the Edman degradation reaction. This method is able to sequence proteins up to about 50 amino acids. If the protein is longer, it must first be cleaved in several places by different peptidases and the individual fragments sequenced and the total protein sequence constructed from this information. The reaction itself consists of taking a pure sample and first breaking any disulfide bonds, if present, purifying the individual chains and absorbing them onto a solid surface. The Edman reagent, phenylisothiocyanate, is then added to the attached peptides with a basic solution that reacts with N-terminal amino acids. This terminal amino acid is then cleaved by the addition of anhydrous acid. The detached amino acid is washed off and identified by chromatography. This cycle is repeated but the efficiency of each step is less than 100% preventing the reliable determination of peptide chains longer than approximately 50 amino acids. Mass spectrometry, however, can theoretically sequence any size of protein. In this method, the protein is digested using an endopeptidase and then passed through an HPLC. After passing through the column, the charged solution is sprayed into a mass spectrophotometer. The charged droplets contain fragmented single ions. The peptides are then fragmented and the mass–charge ratios of the fragments are measured. The resulting spectrum is analyzed with a computer program and compared against other known protein sequences within the database in order to determine the sequence of the fragments. Running a sample multiple times using different enzymes that cleave the protein at various regions allows the sequence of the protein to be constructed according to overlaps from the different peptides.

If the gene sequence is available for the protein in question, the amino acid sequence can be deduced from this information, but for some applications, such as identifying unknowns from an isolate or protein spots from 2D gels, protein sequencing is invaluable.

Cloning

The process of cloning is to make an identical copy of something of biological origin. In molecular biology, this typically refers to multiple copies of DNA, cells, or organisms. Here, we briefly describe certain enzymes used in molecular research and techniques for the cloning of a DNA sequence into a plasmid. Once cloned, the DNA fragment can now be amplified from an expanded culture, expressed, transcribed into RNA, or utilized in a variety of reactions.

Restriction Digestion

Restriction digestion is a method that utilizes restriction endonuclease enzymes. These enzymes are isolated from different bacteria that recognize and cleave specific sequences, thereby cutting DNA into smaller fragments. Restriction endonucleases recognize and cleave specific sequences of between 4 and 8 bp. More than 600 restriction endonucleases are commercially available. Different restriction enzymes will cleave double-stranded DNA at specific base sites located throughout the sequence, each leaving distinctly different terminal ends. Restriction enzymes leave sequence ends

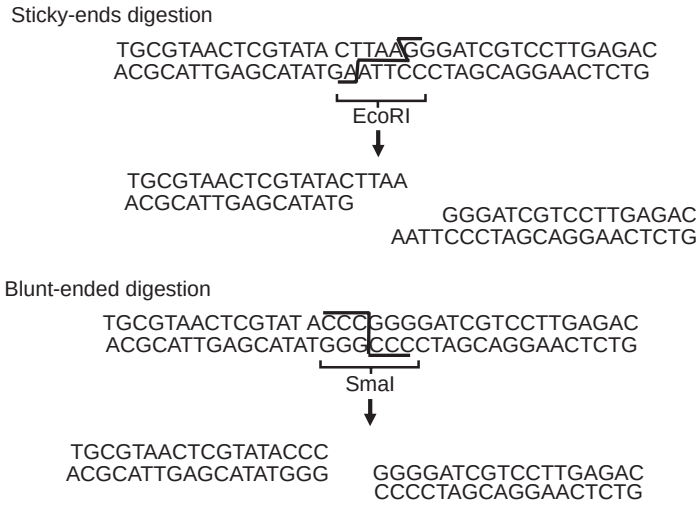

Figure 2.3. Cleavage of double-stranded DNA sequences shown after digestion with a restriction endonuclease. (a) An enzyme that recognizes a particular sequence and after cleavage leaves a 5′ overhang, also called a sticky end. (b) An enzyme that recognizes a different specific sequence and after digestion leaves blunt-ended fragments.

that are either uneven in length between the two strands (overhangs) or equal length (blunt) after cleavage (Figure 2.3). These precise cuts and base-specific ends are useful for cloning or for making restriction fragment length polymorphisms (RFLP). When cleaving large pieces of DNA such as whole genomes, the size of the recognition site for the restriction enzyme determines the relative size of expected digested fragments; so, assuming a sequence to be totally random (50% G + C), a four-base recognition site occurs 4^4 or every 256 bases, while an eight-base recognition site would be recognized and cleaved on average every 65,536 bases (4^8). Therefore, specific restriction enzymes are useful for cleaving large pieces of DNA into smaller sizes, which can then be separated on gels, or cloned into conventional cloning vectors.

Other Modification Enzymes and Their Uses

There are a number of other enzymes useful for the modification of nucleic acid substrates for different assays. Some are involved in sequence conversion such as RNA to DNA, attaching a label to a probe sequence, inserting a sequence into a vector, or modifying fragment ends to prevent self-ligation. Below is a list and description of several of the most common enzymes and some of their uses in molecular techniques.

As the name implies, the primary function of polymerases is the formation and elongation of RNA or DNA against a complementary template. Specifically, polymerases take available nucleotides from a solution and catalyze the synthesis of a sequence. The DNA polymerase Taq from *Thermus aquaticus* is able to withstand high temperatures, thereby allowing it to survive the high-temperature conditions necessary for the multiple denaturation steps that occur during several cycles of PCR. Besides Taq, the enzymes Pfu (*Pyrococcus furiosus*) and Vent (*Thermococcus litoralis*) have also been cloned and are used for PCR. Klenow is a fragment of DNA polymerase I. It

retains the desirable properties of DNA polymerase I and can be used to synthesize DNA from double-stranded templates, fill in recessed 3′ ends, and digest protruding 3′ overhangs. Because of these properties, Klenow is primarily used for labeling DNA probes. T4 polymerase is another enzyme that is also used in similar reactions to Klenow. Reverse transcriptase is an RNA polymerase and is used in the conversion of RNA into double-stranded complementary DNA (cDNA). Other polymerases such T3 and T7 are used for the in vitro transcription of RNA from DNA sequences cloned into plasmids. Terminal transferase catalyzes the addition of nucleotides to the 3′ terminus of linear DNA. This enzyme is useful for the labeling of 3′ DNA ends and adding complementary homopolymeric tails to DNA. Nucleases are typically hazardous for nucleic acids; however, two nucleases that are both purified from bovine pancreas are indispensable for some molecular biological reactions. Deoxyribonuclease I (DNase I) cleaves double- or single-stranded DNA and is used for the removal of DNA from RNA isolations, DNase footprinting, and labeling of DNA fragments by nick translation. Ribonuclease A is an endoribonuclease that cleaves single-stranded RNA. It is used in removing RNA from isolated DNA and in mapping mutations via mismatch cleavage analysis. Exonuclease I is an enzyme that is often used to digest single-stranded oligonucleotide primers following PCR to prepare amplified DNA fragments for sequencing. Ligase is an enzyme that is used to connect fragments of DNA that have corresponding complementary ends. Obviously, ligases are used for cloning inserts into vectors and attaching linkers (short sequences used to modify the ends of existing sequences). Other enzymes such as kinases and phosphatases either attach or cleave a phosphate from the end of a nucleic acid sequence. Phosphatases catalyze the removal of the 5′ phosphate residue. This is useful to prevent self-ligation of compatible ends during cloning and to remove the phosphate prior to end labeling. Calf intestinal phosphatase and bacterial alkaline phosphatase are two such enzymes. Kinases, such as T4 kinase, on the other hand add a phosphate to the end of DNA fragments and are used to generate end-labeled probes.

Methods

Vectors

Typical cloning consists of ligating fragments of DNA into plasmid cloning vectors. Plasmid vectors are circular episomal fragments of bacterial chromosomal DNA that are capable of replicating independently. Most plasmid vectors contain a replicator, a selectable marker, and a cloning site. The replicator allows the plasmid to propagate individually within a bacterium and the selectable marker is usually a gene for antibiotic resistance. The cloning site is cleaved by restriction digestion and the two free ends of the DNA fragment are ligated directly to the complementary cleaved free ends of the plasmid. The area where the plasmid is cut to allow cloning is referred to as a multiple cloning site (MCS). The MCS is a designed region within the plasmid sequence that can be cleaved by multiple different restriction endonucleases to facilitate cloning of DNA fragments without interfering with the plasmid's ability to replicate or confer antibiotic resistance. Once the DNA is ligated into the plasmid, the plasmid is moved into a bacterium by a method called *transformation*. The transformed bacteria are then grown in media containing an antibiotic. If the DNA fragment and the multiple cloning region of the plasmid were both cleaved with two

different nonblunt-end-generating restriction enzymes, then only those plasmids that have had the DNA fragment of interest cloned into them will be circular and able to replicate and confer antibiotic resistance to the bacteria, thus allowing for it to grow in the antibiotic containing media.

Alternative methods of selection depend on the presence of other reporter genes such as the x-gal/*lacZ* system, or green fluorescence protein, which allow selection based on color and fluorescence, respectively. After the DNA fragment of interest is confirmed cloned into the plasmid and bacteria containing that clone are isolated, the bacteria can be grown in large batches and large amounts of the plasmid harvested. Multiple methods of selection can be incorporated to assist in selecting bacterial clones that contain correctly cloned sequences. Once a DNA fragment is cloned into a plasmid, not only can it be amplified, but depending on the specific type of vector the cloned sequence can also be in vitro transcribed to make RNA or protein. Production of proteins requires that the fragment is cloned into an expression vector. Most plasmids can only accept DNA fragments of about 1–10 kb. To clone larger DNA fragments, lambda phage, cosmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), P1-derived artificial chromosomes (PACs), or human artificial chromosomes (HACs) need to be used.

Expression Cloning

Another important use for plasmids is the production of large amounts of specific proteins. In this case, researchers first grow bacteria or eukaryotic cells containing a plasmid harboring a gene of interest. In the same way, bacteria produce proteins to confer antibiotic resistance; cells can also be induced to produce large amounts of proteins from cloned genes regulated by specific promoter sequences. Such proteins are called recombinant proteins. In instances where the protein is produced in low quantities or hard to isolate by other methods, having a protein expression vector produce the protein can be a cheap and easy way of mass-producing a gene transcript or protein. Specific examples include insulin, human growth hormone, interferons, chymosin, and phytase (Walsh 2002). The specific protein being expressed and the purpose of the expressed protein need to be considered when conducting this procedure. If the cloned sequence is of eukaryotic origin then the differences between microorganisms and eukaryotic cells need to be taken into account. For instance, at the transcription level, one must consider the inability of prokaryotes to deal with the presence of introns and the possible effects of DNA methylation. In order to be functional or even display specific epitopes, certain posttranslational processing not found in prokaryotic cells is sometimes necessary. Such posttranslational modifications to the protein include phosphorylation, sumolation, or glycosylation and may be required for proper structure and function.

Gel Electrophoresis

General

Gel electrophoresis is a technique used to separate nucleic acids or protein molecules by size, using an electric current applied to a gel matrix. It can be performed for

analytical purposes such as identifying fragment sizes after restriction endonuclease digestion similar for RFLP analysis or PCR or as a method of preparing samples for other downstream applications. Electrophoresis of nucleic acids separates sequences by weight so that individual fragments can be determined and sized, and then sequences can be directly isolated from the gel, or the fragments can be further analyzed by other methods such as Southern blotting. For basic analysis of DNA, the most common gel matrix used is made of agarose. The sugar phosphate backbone of nucleic acids is negatively charged and in a gel it will migrate from a negative to a positive electrode. Typically, linear and supercoiled DNA fragments are run in simple agarose gels in a salt buffer. RNA, however, because of hydrogen bonding tends to fold and migrate through gels in a complex manner unless a disruptor of the bonds such as formamide is added to the gel. Proteins also need to be run in a gel containing a denaturing agent, usually a detergent such as sodium dodecyl sulfate (SDS). The detergent removes the tertiary structure of the protein and gives it an overall negative charge, allowing the protein to migrate through the gel according to its size. Instead of agarose, most protein gels use a matrix made from polyacrylamide (PAGE). Protein gels can be used to determine protein concentrations or for the separation and identification of specific proteins present in a sample. Polyacrylamide gels are also used to separate and detect single base pair difference in DNA fragments. Pulse-field gel electrophoresis (PFGE) is a special method used to separate very large fragments of genomic DNA larger than 20 kb. The development of this technique was required because pieces of DNA larger than 20 kb would all migrate through a standard agarose gel at the same speed. PFGE is basically done by applying an alternating voltage gradient to the samples in the gel to aid in their separation and provide greater resolution. Two-dimensional gel electrophoresis is a technique that is applied to proteins to enhance the isolation of discrete proteins. On a regular gel, the proteins are isolated by charge alone, if many proteins are present in the sample then usually multiple proteins will migrate together or will be so close that it is relatively impossible to isolate a single pure protein from a gel. Then, the proteins can usually be separated by 2D gel electrophoresis (discussed in more detail in Chapter 7). With this method, the proteins are separated for two distinct properties such as mass and isoelectric point (pH at which a protein possesses no net electrical charge). This method is being used to look for proteins that are differentially regulated under specific conditions. Then, when gels are run on isolated protein extracts obtained from control and experimental animal tissues, differentially expressed proteins can be identified, isolated, and sequenced to distinguish the activity of the protein.

Molecular weight markers for both nucleic acids and proteins are available and typically run on the gel to aid in determining the size of individual bands present.

Polymerase Chain Reaction

PCR is a method for the exponential amplification of DNA. All modern PCRs require a thermostable polymerase that is able to survive and function after being heated for several cycles. With this technique, it is possible to amplify only a few copies of sequence by several orders of magnitude. A PCR consists of a DNA sequence; a thermostable polymerase; two oligonucleotide primers complementary to the DNA sequence; a deoxynucleotide triphosphate mix containing all four bases, adenosine, guanosine, thymidine, and cytosine; and a buffer typically containing the

divalent cations magnesium (Mg) or manganese (Mn). Reaction volumes can be run in as little as a couple microliters in 96 or 384 well plates or even less with some of the newer microfluidic plates available (Khandurina et al. 2000). The samples are then subjected to cycles with multiple temperature changes. Each cycle consists of denaturation, annealing, and elongation steps that are typically repeated between 15 and 40 times. In the denaturation step, the sample is heated and held at a high temperature (~95°C) for 5–30 seconds to separate the two strands of the DNA molecules by disrupting the hydrogen bonds holding the strands together. Then in the annealing step, the sample is cooled to allow the two primers to anneal to the now accessible complementary sequence on each strand of the now single-stranded DNA. This step occurs at temperatures between 45 and 65°C and takes place for 15–40 seconds. The elongation or extension step is the final stage of the cycle during which the polymerase uses the free nucleotides in solution to extend the primer sequence, making a double-stranded piece of DNA. This step can be anywhere from 15 seconds to more than 2 minutes depending on the size of strand being amplified. The amplified fragment can then be visualized on a gel, cloned into a vector, or used in any number of applications. Real-time quantitative PCR is being used extensively in many research laboratories, and it is discussed in more detail in Chapter 3, but basically it is a method whereby PCR is now used to quantify the presence of a specific sequence.

Microarray

Microarrays are a method for detecting relative changes in the level of a nucleic acid or protein by hybridization of prepared sample material against a slide or nylon membrane containing hundreds or thousands of spots of complementary sequence or antibody. Designs and platforms used for the fabrication of microarrays are covered in Chapter 4. Each spot on a microarray contains multiple copies of a single substrate, typically cDNA, oligonucleotides, or proteins, that are attached to the slide and which bind specifically to one nucleic acid or protein sequence under certain hybridization conditions. The arrayed spots can be covalently bound to glass, nylon, and similar membranes. Experimental sample material, either cDNA or protein, is labeled (usually with a fluorescent label) and hybridized to the spots on the microarray. After several washing steps, the microarray is visualized to determine the relative fluorescent intensity of all the spots. Spot intensity is then used to determine relative changes in level of sample cDNA or protein between two different groups, typically an experimental and control group. DNA-binding arrays may be used to evaluate differences between distinct genomes or to look for alternative single nucleotide polymorphisms or splicing sites. Protein arrays are useful for the screening of protein–protein interactions, for the identification of biologically active compounds, and for the identification of protein activity sites such as GTP binding or phosphorylation sites. However, most of the arrays published to date have been used to examine gene expression. This technology has a tremendous potential for the acceleration of research.

Suppression Subtraction Hybridization

This is another technique that is often used to identify transcripts that are differentially expressed between experimental and control samples, or used to identify genetic

differences between different strains and species. The method relies on the normalization and hybridization of common transcripts, converted into cDNA, between samples. Then with a subtraction step, these sequences are removed, thus leaving behind cDNAs of transcripts that are either more highly expressed or rarer in one population or the other. These cDNAs are then cloned and amplified for further study.

Random Labeling, End Labeling, and Nick Translation

These are all methods for labeling DNA probes. Labeled probes are used in many molecular applications such as Southern blots, in situ hybridization, sequencing, microarray probing, and chromosome labeling. Each technique is a means for the incorporation of a labeled nucleotide into a sequence for the purpose of enhancing the detection of the sequence when it is later used to bind complementarily or is just being run on a gel. Random labeling is a method of incorporating radioactive or fluorescent-tagged nucleotides along the length of a DNA fragment. The double-stranded sequence to be labeled is denatured and random oligonucleotides anneal to both strands. A polymerase such as the Klenow fragment of polymerase I is then used to extend the primers using a mixture of nucleotides of which one has been labeled. This then creates a uniformly labeled double-stranded probe.

End labeling is typically used when small fragments of DNA need to be labeled or only a specific end of a fragment needs to be labeled. The enzyme T4 kinase is often used for the addition of labeled radioisotope or similarly labeled phosphate to the 5′ termini or end of a DNA fragment. End-labeled synthetic oligonucleotides are useful for numerous applications such Maxam–Gilbert sequencing, DNase footprinting or protection assays, and labeling of DNA fragments as probes.

Northern and Southern Blotting

Northern and Southern blots are routine methods for the evaluation of specific sequences from RNA or DNA samples. Southern blots get their name from Edwin Southern, the developer of the technique. For Southern blots, DNA samples are run on gels and then blotted onto nitrocellulose or nylon membranes so that the DNA bands from the gel are now transferred to the membrane. The DNA is then covalently attached to the membrane by baking or UV cross-linking. The membrane with the attached DNA is probed with chemifluorescent or radioactive-labeled RNA or DNA sequence, which hybridizes to complementary sequences on the blot. After the hybridization step, the blot is washed with salt and detergent in solutions with increasing stringency to remove all residual nonbinding probe. The blot is then analyzed by visualization using a CCD camera or upon exposure to autoradiography film. The Northern blot is basically the same as the Southern blot except RNA samples are run in the gel and affixed to a membrane. Samples for both techniques are typically run on agarose gels with formaldehyde added as a denaturant for RNA samples. For the separation and analysis of small fragments polyacrylamide gels are used. Another variant of Northern blot is called the reverse Northern blot. In this case, isolated DNA fragments are run on a gel and then probed with labeled total extracted RNA from a tissue of interest. Dot and slot blotting are techniques similar to Southern blotting

used for immobilizing bulk unfractionated DNA onto membranes for hybridization analysis to quantify relative abundance of target sequences.

Genomic and cDNA Libraries

In molecular biology, the term *library* is used to denote a collection of RNA or DNA molecules. A genomic library represents the entire genomic sequence encompassing all the chromosomes of the species from which it was isolated. The sequence is broken into smaller fragments that can be packaged into a specific vector such as BACs. The main concern with genomic libraries is that some fragments or sequences exist as only a small fraction of the total isolated DNA. For example, a 3-kb fragment represents less than 1 millionth of a normal vertebrate genome. Therefore, in order to ensure the complete representation of all sequences that might be of interest, it is necessary to consider the size of the genome and the size of the fragments that will be cloned from it. In general, to have a 99% chance of isolating a desired sequence, the number of clones contained in vectors should represent greater than a 4.6-fold excess of the total number of base pairs in the genome (Seed et al. 1982). Vectors such as BACs can receive fragments between 100 and 300 kb; therefore, it would be necessary to generate a library containing between 15,000 and 45,000 BAC clones to obtain a library with sufficient coverage to screen for all genomic sequences. More on genomic libraries and their uses along with traditional methods of mapping and markers is discussed in Chapter 5.

A complementary library represents all the genes that are transcribed in a particular tissue that was isolated under specific physiological, environmental, and developmental conditions. Libraries of cDNA are used for expression analysis and contain RNA that has been converted to cDNA using the enzyme reverse transcriptase. These libraries are much smaller than genomic libraries as they only represent actively transcribed sequences that make up less than 1% of the genome. Most full-length transcripts are less than 10 kb, so the obtained cDNA fragments are typically cloned into bacterial plasmids. How the library is screened dictates the type of vector used for cloning inserts. cDNA libraries can be screened by hybridization or by antibody or protein activity if the library is expressed. Quality of the RNA used for library generation and the relative abundance of clones of interest are the major concerns involved in generating cDNA libraries. The highest quality mRNA needs to be used to ensure cloning of sequences with low abundance will be present. Furthermore, for the detection and evaluation of low abundance clones the library needs to have a coverage of at least fivefold greater than the total number of recombinants as indicated by the lowest abundance estimate. Relative cloning efficiency is important for the generation of both genomic and cDNA libraries. A number of cDNA libraries have been generated and are currently being used in the generation of microarrays, which is discussed in detail in Chapter 4.

Mutagenesis

In molecular studies, there are often times when a researcher is interested in how modification at the level of the sequence can affect the activity of a specific gene product.

Changes in sequence might affect phosphorylation sites, binding activity, membrane channel formation, or enzymatic kinetics. There are methods for the generation of random mutations by exposing genomic material to radiation or chemicals, or with PCR by reducing the fidelity of the enzyme. Random mutations are good for the generation of lots of mutants with multiple sequence changes throughout the genome. These mutants can then be screened and the genes identified that have a role in specific physiological differences. In vertebrate research studies, there is substantial interest in determining the effects changes that occur when specific sequences or nucleotides are changed. Through the use of site-directed mutagenesis, short discrete sections of genetic code can be changed in any way desired. There are several different approaches but these are all based on the basic premise of using an oligonucleotide that contains the desired mutation(s) and annealing it to a plasmid containing the complementary strand with the sequence from which the mutation oligonucleotide was derived. The mutant oligonucleotide now serves as a primer for DNA synthesis (Figure 2.4). Using a polymerase, such as T4, which possesses a number of desirable qualities such as a lack in exonuclease and strand displacement activity, a complementary copy of the plasmid is generated. Ligase is then added to seal nicks between the newly generated strand and the oligonucleotide. The plasmid is now a double-stranded heteroduplex with one strand containing the original sequence and the other the mutated sequence. This is propagated in *Escherichia coli* under special conditions to selectively propagate the plasmid containing the mutant sequence. Examples of methods include the use of antibiotics for positive selection of mutated sequence containing plasmid, using a parent strand that contains uracil which can be selectively degraded, cleavage by a restriction endonuclease at a site in the plasmid and subsequent ligation of an oligonucleotide containing the mutation commonly referred to as cassette mutagenesis, and incorporation of analog nucleotide bases that can render the mutant strand endonuclease resistant.

Site-directed mutagenesis can also be accomplished by PCR using oligonucleotide primers that contain the desired mutation. Many variations exist; however, the general basis of these is that as the polymerase uses the primer to amplify the sequence it now is incorporating that sequence into the newly synthesized strands (Higuchi et al. 1988). In theory, after multiple cycles the vast majority of amplified product should contain the correct mutant sequence, the original template sequence is methylated and can be removed by digestion with Dpn1 that cleaves only the methylated template DNA and not the unmethylated amplified mutant sequence. PCR-directed mutagenesis is a fast and relatively simple method; however, typical thermostable polymerases lack $3' \rightarrow 5'$ proofreading capabilities and can potentially introduce a number of secondary mutations. Therefore, extra sequencing may be required to ensure that only the desired mutation is contained in the PCR-generated sequence.

Antibodies

Antibodies are produced by the immune system for the purpose to recognize invading organisms or other "nonself" material. Due to a region known as the hypervariable region, antibodies possess the capability to specifically recognize and bind to all antigens. The antigen is typically some structure, termed an epitope, of a protein or polysaccharide that is recognized as foreign and stimulates antibody production. In

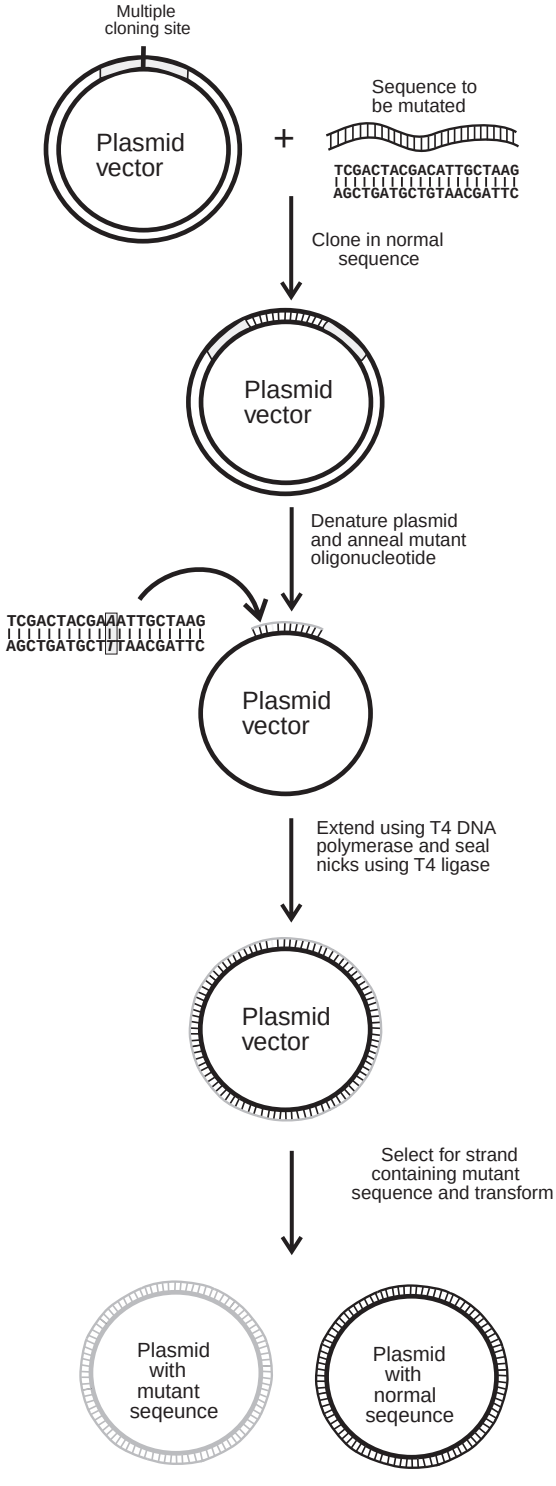

Figure 2.4. Schematic outline for generation of site-directed mutagenized sequences.

molecular biology, antibodies are useful for many applications involving the identification and location of proteins in cell or on blots. Antibodies are produced by B cells in the body. When a foreign object, such as bacteria, invades the body, these cells will produce a specific antibody to every epitope available. Therefore, hundreds of different antibodies can be produced and each is specific for some epitope of the bacteria. In research, the antibodies harvested from the sera of an appropriately injected animal, such as a rabbit or goat, are called polyclonal antibodies. The B cells of these injected animals produce IgG immunoglobulins that are specific for the injected antigen. From sera harvested from these injected animals, polyclonal IgG is purified. A researcher can now concentrate the antibody to high titers for use. In general, larger proteins are better antigens and produce more specific antibodies. These work well for enzyme-linked immunosorbent assays (ELISAs) and Western blots. Antibodies can also be produced that specifically recognize only one epitope on an antigen. These are called monoclonal antibodies, and these are produced as clones from an individual antibody-producing cell harvested from an injected mouse or rabbit fused with myeloma cells. Selection media is used to select for hybridized cells called hybridomas. Individual cells are isolated and then grown and tested for their affinity to bind with the antigen. Hybridoma cells are able to grow indefinitely and continually produce a fluid filled with the antibody. Monoclonal antibodies also work well with Western blots and ELISAs but also are used to purify antigenic material through immunoprecipitation and affinity chromatography. Many polyclonal and monoclonal antibodies are commercially available. Depending on what animal cell the antibody is produced from, there also exists an antibody against these animals' cells that are used to attach and amplify a signal from the protein-specific antibody being used.

Western Blots and ELISAs

Western blots are similar to Southern and Northern blots except in this case a protein is being run on a gel, blotted, and the separated protein bands are affixed to a membrane and probed. For Western blots, protein extracts from tissue are separated by gel electrophoresis on normal or denaturing PAGE gels. The separated proteins are then electrophoretically blotted onto a membrane and probed with antibodies. A typical application is for the detection or relative quantification of a specific protein in an isolated tissue sample.

ELISA is a technique used for the detection of antibodies or antigens from a sample. Whereas Western blots are run on gels, ELISAs are run on multi-well plates. A basic ELISA consists of affixing an unknown amount of antigen to the surface of a well; serum is a commonly used sample, then an antibody is added that attaches to a specific affixed antigen and finally an enzyme substrate is added for detection. Originally, detection was almost exclusively done using radioactive-labeled antibodies, radioimmunoassay, but now most assays use an enzyme such as horseradish peroxidase, which causes a color change when its substrate is added. Fluorescent-labeled conjugates are also used. The methods for performing ELISAs follow the previously described premise but differ in the methods of the order and how the conjugates are assembled in the well. Direct ELISAs affix the unknown material directly to the plate and then attach an antibody that may or may not be linked to a detection enzyme. These assays are simple and direct requiring only one antibody (Figure 2.5a). Indirect

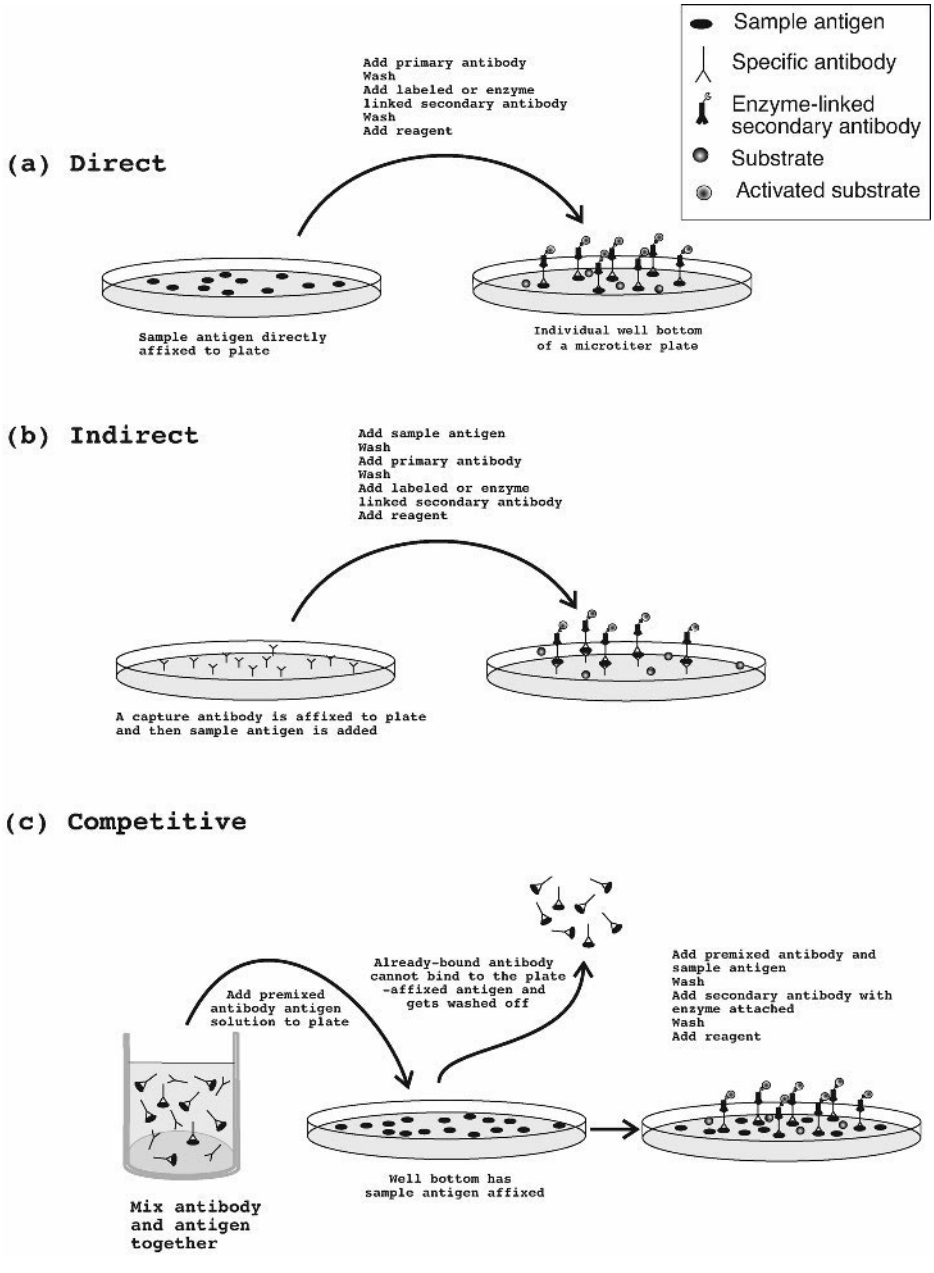

Figure 2.5. ELISA methods. (a) Direct ELISA whereby the antigen is directly affixed to a plate surface and then probed with a primary antibody. (b) Indirect ELISA in which a capture antibody is affixed to the plate surface and then incubated with an antigen and finally detected upon incubation with another antibody. (c) In the competitive ELISA the antibody and antigen are premixed, incubated in a well with an antigen-coated surface, and then washed and the well is probed with a secondary antibody specific to the primary antibody.

ELISAs are used primarily to determine the strength and quantity of an antibody from serum samples, usually from immunized animals or hybridoma supernatants. A known antigen is first affixed to the bottom of the well, next the serum is added, and finally a secondary labeled antibody, anti-rabbit or anti-mouse, is added to bind to the serum antigen. A substrate is added last for the generation of a detectable signal. These types of assays are also referred to as sandwich ELISAs because the antigen being measured is conjugated between the capture antibody affixed to the plate and the detection antibody (Figure 2.5b). A competitive ELISA functions by mixing the sample antigen with a fixed concentration of antibody and then adding this to a microtiter plate where the wells are precoated with antigen. Unbound antibody is able to bind the antigen on the plate while antibody that is previously bound is unable to attach to the antigen affixed to the plate. Therefore, upon washing the well all the prior bound antibody is removed and an enzyme-linked secondary antibody is added for detection. For the competitive assay the greater the level of antigen present in the sample correlates with less free antibody available to bind to the antigen affixed to the well. Thus, the greater the signal from the well correlates with less initial available antigen from the sample (Figure 2.5c). Oftentimes, a labeled secondary antibody against the IgG of the animal from which the primary antibody was made is used because a secondary antibody can greatly amplify the signal and it is also more economical than having to purchase or make labeled primary antibodies.

In Situ Hybridization and Immunohistochemistry

These are methods used for the localization and relative quantification of mRNA transcripts or protein directly in tissue sections (Jin and Lloyd 1997). With in situ hybridization, the isolated tissue is treated by either freezing or embedding it in paraformaldehyde to preserve and fix the transcripts in place. Then, a probe that can be labeled with either radioactivity, fluorescence, or an antigen is hybridized directly to the transcript affixed in the tissue. So, as with blots, the sectioned tissue is washed under increasing stringent conditions to remove the probe, and then visualized by either autoradiography, fluorescence microscopy, or immunohistochemical means. Immunohistochemistry uses antibodies to detect specific proteins in tissue sections. Like with other immunological assays, monoclonal or polyclonal antibodies are used and can be either directly labeled or the label is conjugated to a secondary antibody specific for the IgG of the animal from which the primary antibody was produced. And like with in situ hybridization, the same labels and detection systems are used (Ramos-Vara 2005). Both techniques typically take quite a bit of refinement to obtain proper binding and hybridization conditions and reproducible results.

Protein Interactions

DNA–Protein Interactions

DNase footprint analysis is designed to determine the specific binding sites of proteins on DNA. The enzyme DNase I is used to cleave end-labeled DNA fragments, which are then run on a gel for analysis of the resulting cleavage pattern by autoradiography.

DNA bound to protein is protected from enzymatic cleavage, resulting in a clear area on the gel that is referred to as the footprint. By varying the concentration of the DNA-binding protein, this technique may be used to generate binding curves and to determine the binding affinity of proteins at individual binding sites.

The electrophoretic mobility shift assay is another technique for studying gene regulation and determining DNA interactions with proteins. This assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is carried out by first incubating a partially purified protein or protein extract with a ^{32}P end-labeled DNA fragment that contains the expected protein-binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel or agarose gel if large fragments are being studied. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences. Furthermore, when a complex mixture of proteins is being analyzed, this assay can also be modified into what is called a supershift assay. This is used when the level of proteins in the mixture generates an excessive number of bands, thus making it unclear which protein is binding to the DNA. If an antibody is available that interacts with a protein of interest, one can ask whether a particular shifted band contains the protein by having a second incubation that includes the antibody. If the complex shifts further up in the gel, this is evidence that the antigen was present in the initial complex and the reason for the increased shift is that the complex now also contains the antibody.

Another method used to evaluate DNA and protein-binding sites on genomic DNA for a protein of interest is by using chromatin immunoprecipitation. With this technique, proteins binding to the chromatin structures inside the nucleus are cross-linked in vivo. Then, the cells are lysed and the DNA is broken into small pieces less than 5 kb. An antibody specific to the protein of interest is then used to immunoprecipitate the chromatin-bound protein. The protein is released and the sequence of the DNA fragment is determined (Stahl-Bolsinger et al. 1997; Evans et al. 2000).

Protein–Protein Interactions

The interactions between proteins are of interest because of the many physiological functions where proteins play a direct role, such as in signal transduction, metabolism, cellular control, growth regulation, and almost all regulatory events in a living cell. These interactions can be through the binding or activity of a single protein, homo-oligomers, or large hetero-protein complexes. Because of the many diverse interactions, there have been a number of complicated and highly variable techniques developed for the analysis of protein–protein interactions. Only a few of the techniques will be briefly discussed and individuals interested in more in-depth information should refer to the references listed.

Coimmunoprecipitation is one of the more common methods for demonstrating interactions and involves binding an antibody to a specific protein and running this with whole cell extracts through a column. The antibody attached to protein is fixed in the column along with any other proteins attached. The residual material is washed away and then the protein of interest and any attached proteins are eluted and then

identified by Western blotting or protein sequencing. To identify protein complexes that contain a large number of proteins, multiple antibodies and rounds of isolation are usually necessary (Michielsen et al. 2005; Howell et al. 2006).

The yeast two-hybrid screen uses the interaction of two fusion proteins, one containing a DNA-binding domain and the unknown proteins of interest are attached to an activation domain. Both proteins must then bind to stimulate the transcription of a reporter gene (Fields and Song 1989; Fields and Sternglanz 1994).

Phage display is a high-throughput screening method, whereby a library of DNA sequences is ligated to a bacteriophage gene and then transformed into bacteria. This is then expressed, and the proteins produced are incubated with protein targets immobilized and attached proteins are eluted. This is repeated to generate an enriched population of proteins of interest, which can then be isolated, amplified, and each specific isolate analyzed (Kay et al 2001; Willats 2002).

Another method involves the isolation of proteins that have been cross-linked in vitro or in vivo. Therefore, the cross-linking is performed in the tissue, and any interacting proteins isolated leads to the potential isolation of many different protein complexes. Or protein extracts can be cross-linked to specific proteins already affixed to a surface. Many cross-linking reagents are available, and some can be cleaved once the proteins are isolated to facilitate analysis of the individual proteins. The different cross-linker reagents tend to differentially bind proteins using different cross-reactive agents and also being selective for the distance between the proteins (Phizicky and Fields 1995; Vasilescu et al. 2004; Suchanek et al. 2005).

Another method is based on the previous discussed Western blot, but in this case instead of probing with an antibody, a nonantibody protein is used to detect binding. A tag or label attached to the probe protein allows for visualization of the bound protein, alternatively if an antibody exists for the probe then it may be used (Phizicky and Fields 1995; Ulrich et al. 2001). Alternatively, there are techniques such as surface plasmon resonance (SPR) that may be summarized as the detection of the biospecific adsorption of a protein attached to another protein in continuous flow. In SPR, a known protein is affixed to a suitable metal surface and the change in refractive index is used to measure the binding of proteins in a label-free manner. Other than the refractive metal surface that allows proteins to be covalently bound to it, this technique requires an integrated fluid system to pass the material over the covalently attached proteins and biocompatible sensor chips. The response signal from the SPR detector is proportional to the mass and surface area of the protein (Stenberg et al. 1991; Szabo et al. 1995).

Fluorescence resonance energy transfer, described in more detail in Chapter 3, is a useful means to measure the thermodynamics and kinetics of protein interactions. Two proteins being studied for their binding interaction can be labeled with fluorescent dye molecules, and changes in proximity between the proteins can be measured by observing the transfer of energy between the two chromophores (Rye 2001; Cristea et al. 2005).

Conclusion

This concludes this brief section delving into some of the varied reagents, methods, and techniques used by molecular biologists to discover how differences at the genome

are transferred into detectable physiological variation. The reader should note from reading through the techniques in this chapter that within the cell there are many different levels, including genomic, transcriptional, translational, cytoplasmic, organelle, or cell membrane, at which a reaction can be studied. And whether determining the genetic variation between individuals or specific cellular mechanisms controlled by the genes involved, devising appropriate experiments, and employing the proper techniques to study the event are the primary basis for understanding the physiological implications.

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Chapter 3
Quantitative PCR

Ken Overturf

Introduction

A significant amount of the current basic aquaculture research being published is focusing on the expression of specific genes to better understand certain physiological events. Research evaluating the genes involved in development, both embryonic and sexual, growth, disease resistance, and nutrient partitioning is generating a substantial body of knowledge that has significantly enhanced out current understanding of these biological activities in fish. This chapter first seeks to provide the reader with the basic information necessary to understand the intricacies of quantitative polymerase chain reaction (qPCR) and then describes certain aquaculture studies and the findings to date from their analysis of gene expression in fish. Further demonstrations of quantitative polymerase chain in aquaculture research are provided in specific detail in several of the following chapters.

qPCR analysis is a method for using the exponential amplification of sequence by PCR to quantify the level of an mRNA message or DNA sequence present in a sample. This method has gradually evolved from standard PCR practices. Basic PCR is now considered an essential molecular procedure in all research laboratories for the amplification and detection of nucleic acids and for genotyping, sequencing, and cloning. qPCR is a modification of the PCR, whereby the actual or relative copy number of the initial product is determined either during the reaction run, such as with real-time (RT) qPCR, or at the end of the reaction as with semiquantitative PCR. Like standard PCR, the qPCR functions by using the temperature-mediated enzyme DNA polymerase for sequence amplification. But with qPCR the accumulation of amplified sequence, also termed amplicon, is now being quantified directly during the PCR. Semiquantitative PCR, alternatively known as end-point analysis PCR, quantifies amplified product postreaction utilizing methods such as gel analysis, detection of radioactive label, or HPLC, while in the case of RT-qPCR, accumulation of amplicons is quantified every cycle of the reaction through the use of labeled primers, probes, or intercalating dyes.

The abbreviation RT for real time in quantitative RT-PCR should not be confused with the abbreviation for reverse transcription, which is a step often used in qPCR that involves the conversion of RNA to complementary DNA (cDNA) through a specific enzymatic reaction. The two methods may be used in concert with reverse-transcription of mRNA and then quantification of the resulting cDNA using RT-PCR (also referred to as quantitative RT-PCR). To avoid confusion in this discussion, RT-PCR denotes real-time PCR while qPCR represents quantification of total amounts of specific sequence using real-time or semiquantitative PCR. Any other descriptions or denotations are spelled out at the time of their usage.

qPCR Basics

qPCR is used in a wide array of scientific research areas, including, but not limited to, developmental biology, microbiology, physiology, immunology, and a majority of other studies evaluating the genetic effect of physiological changes or when attempting to determine the copy number or presence of a specific genome (Bustin 2000). As described previously in Chapter 2, PCR, through the use of complementary primers that bind to DNA and with the action of a polymerase, amplifies a specific sequence whose copy number theoretically doubles every cycle during a reaction run (Mullis 1990). Originally, qPCR or what is now referred to as semiquantitative PCR was performed by running a PCR reaction, and then analysis occurred by quantifying band intensity on agarose gels or by other postamplification means. Semiquantitative PCR requires rigorous controls to restrict for potential calculation errors that might be induced due to factors such as differences in polymerase fidelity between samples, measurements taking place outside the exponential amplification phase, and other related aspects (Ginzinger 2002). Besides qPCR, other methods used to detect and quantify nucleic acid sequences include Northern and Southern blots hybridizations, high-performance liquid chromatography (HPLC), scintillation proximity assays, PCR-ELISA, RNase protection assays, and in situ hybridization. For many purposes, the Northern blot or the more sensitive RNase protection assay is sufficient for detecting relative quantitative differences between samples. However, most of these methods typically have one or more of the following problems; they may require the use of radioactivity, are time consuming, labor intensive, have a potential for cross-contamination, and are either insufficiently sensitive or not truly quantitative. Since around 2000, RT-PCR has rapidly been replacing semiquantitative PCR in most laboratories, and in the published literature, and is currently the most widely accepted method for the determination of absolute or relative copy number in gene expression, microarray validation, and for the detection and quantification of genomic sequence from isolated samples (Valasek and Repa 2005). Besides the listed negative aspects of quantifying sequence via semiquantitative PCR, there are several other reasons for the rapid increase in the use of qPCR. These include the huge influx of genetic sequence data now available for a multitude of different organisms, advances in DNA and RNA isolation procedures, and the development of several different platforms and chemistries for running samples. Furthermore, other advantages include the development of simple and rapid complete postrun analysis without further steps or manipulation, and improved software for analysis of sequences and generation of primers and probes necessary to perform RT-qPCR (Freeman et al. 1999; Bustin 2002).

The Basic qPCR

Most qPCRs begin with the isolation of nucleic acids, either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), from whole organisms such as viruses or from isolated tissues and tissue culture. There are several accepted methods for the isolation of nucleic acids, which is briefly covered in Chapter 2. If RNA is to be quantified then first the isolated mRNA needs to be converted into single-stranded cDNA. RNA cannot be run directly in a qPCR because it would rapidly deteriorate under the temperature conditions of the reaction, and also a thermostable high-fidelity RNA

polymerase does not exist. So, initially the mRNA must be converted to cDNA and this requires the action of the retroviral enzyme reverse transcriptase. There are several commercial forms of the enzyme available such as Moloney murine leukemia virus, avian myeoblastosis virus, and thermostable reverse transcriptase/DNA polymerase, which may be purchased with any necessary buffers and reagents (Bashiardes and Lovett 2001).

Conversion of mRNA to cDNA can be performed as a separate reaction, quantified and aliquoted for RT-qPCR or the reaction can be combined with PCR and run as one step, whereby after the reverse transcription reaction all the cDNA converted in each well is directly amplified by PCR. For one-step quantitative reverse transcription PCR, the amount of cDNA produced by reverse transcriptase needs to accurately represent the initial RNA input for all samples being compared. Some research has also shown that residual reverse transcriptase can negatively affect PCR efficiency (Chandler et al. 1998; Suslov and Steindler 2005). Reverse transcription reactions are usually carried out between 40 and 50°C, and at these temperatures the potential for errors in site-specific primer annealing can be substantial. Therefore, the thermal range, sequence-binding sensitivity, and specificity of the enzyme need to be considered for every qPCR assay. When the template concentration is extremely low, this becomes an additional problem. Low template concentration increases the potential for nonspecific binding, resulting in the amplification of undesirable products, which can then also result in the complete inhibition of amplification of the desired amplicon. Sequences that are rich in guanine and cytosine or that form stem loops are also a problem during the reverse transcription reaction, as they can cause the enzyme to stop or become separated from the RNA template (Diffenbach et al. 1995).

In order to initiate cDNA synthesis, an oligonucleotide primer of complementary sequence is required. The primer anneals to the RNA, and the cDNA is extended toward the 5′ end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase. Primers can be either gene-specific or nonspecific. Nonspecific or random primers typically are hexamers or hexameric oligonucleotides; that is, they contain all possible nucleotide combinations in a six-base sequence. Another method is to use oligo(dT) primers that consist only of deoxythymidine residues and anneal to the polyadenylated 3′ end tail of the mRNA. Reverse transcription reactions primed by hexamers and oligo(dT) primers will produce a random amplification of all RNA sequences contained in the reaction mixture.

An alternative to random priming is the use of a primer that anneals to a specific sequence. These specific primers are typically for the amplification of cDNA explicit for a region or a gene of interest. In some cases, such as the quantification of rare mRNAs, the use of sequence-specific primers will increase the specificity and decrease the background associated with random primers. Another benefit is that gene-specific primers almost always anneal at higher temperatures, thereby reducing the potential for superfluous transcripts. In one-step qPCR reactions, the primer used in generating the cDNA can be used in the following PCR reaction. However, it must be noted that when specific primers are used in the synthesis of cDNA during reverse transcription reactions then only that gene or region can be quantified in subsequent qPCR reactions from the generated cDNA, while cDNA synthesized with random primers can be used for the quantitation of any gene of interest.

After the cDNA is generated, or if only DNA is being quantified, the next step is amplification of the region or gene of interest. Basic PCR accomplishes this with a

thermostable polymerase and two site-specific primers. Then the amplified products need to be labeled and quantified. Methods for labeling and quantifying the amplified product are what distinguish semiquantitative PCR from RT-qPCR. Also of importance is whether quantification of the amplified product is going to be relative or absolute. Relative quantification correlates the PCR signal of the target transcript in a treatment group to that of another sample, such as an untreated control, and this is typically normalized to a housekeeping gene; for example, β-actin or ribosomal RNA is commonly used as normalization genes (Saunders 2004). Absolute quantification determines the actual input copy number, usually by relating the PCR signal to a standard curve generated with a known copy number of an identical sequence, such as from a synthesized oligonucleotide. Standard curves can be used for absolute or relative quantification. Serial dilutions of nucleic acid material such as plasmids, oligonucleotides, in vitro transcribed RNA, or even concentrated RNA samples are suitable for standard calibration in real-time reactions. The standard curve is generated by performing serial dilutions with a solution containing the standard sequence and assaying each dilution together with positive and negative control reactions. To maximize accuracy, the dilutions are made over the range of copy numbers that include the expected amount of target sequence in the experimental nucleic acid samples. A threshold cycle, the point at which the reaction is determined to be increasing exponentially for semiquantitative PCR or when fluorescence rises appreciably above the background fluorescence in RT-PCR (Figure 3.1), is then determined for each reaction and plotted versus the absolute amount of gene-specific material or the fold dilution of the nucleic acid source. The data are then fit to a straight line yielding a calibration curve, which is used to determine the absolute or relative amount of gene-specific material in unknown samples. The slope of the standard curve should

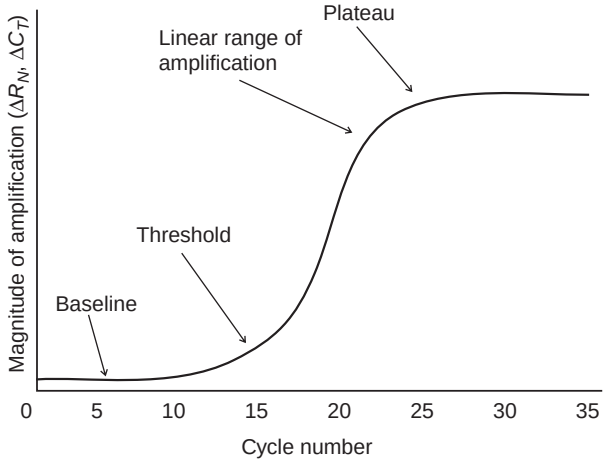

Figure 3.1. The phases of PCR. (1) Baseline phase—where the reaction begins but prior to exponential amplification. (2) Threshold phase—where product amplification begins and is recognized over the background. (3) Linear range of amplification phase—where amplification occurs exponentially, and the doubling of products occurs every cycle. During this phase, there is a direct correlation between cycle number and sequence concentration. (4) Plateau phase—where the reaction has stopped and product is no longer being produced.

be between −3.1 and −3.6 if the PCR amplification efficiency is approximately 100% (−3.32), with the *y*-intercept providing the sensitivity of amplification efficiency. The calculation for estimating reaction efficiency is Efficiency $= 1 + 10^{(-1/\text{slope})}$. The correlation factors (R^2 values) for standards should be at least 0.95 or greater for reliable results (Freeman et al. 1999; Raeymaekers 2000; Pfaffl 2001; Rutledge and Cote 2003). These figures have to be determined experimentally and then determined postanalysis for semiquantitative PCR, while current RT-qPCR systems automatically calculate these data and use them to determine sample quantification immediately post-PCR.

Semiquantitative PCR

As a PCR method for quantification of nucleic acids, semiquantitative PCR was used extensively during the early 1990s, and it is still used to a large degree in many smaller molecular research laboratories. Semiquantitative PCR can be run and analyzed by a number of different methods. The following sections provide a brief overview of the aspects of semiquantitative PCR. In order to quantify initial sample quantity using semiquantitative PCR, it is necessary to ensure that the reaction is stopped during the exponential range of amplification and that a proper method is established for product detection. As shown in Figure 3.1, the exponential range of amplification for a PCR exists when the reaction components are in excess and amplification products are accumulating at a constant rate. The exponential range occurs postthreshold and prior to the linear and plateau phases. Copy number amplification starts to become linear and then plateau during the later cycles of the PCR reaction due to the consumption of PCR reagents such as nucleotides, the generation of inhibitors, or competition of PCR products with primers during the annealing step. There are several different methods used to verify that measurements are taken during the amplification phase. These include running serial dilutions with the template, running multiple reactions, and then stopping the reactions at different times in order to alter the number of amplification cycles, adding a competitor template or the incorporation of similar primers that amplify a different region of the same length into the reaction. The last step then is the evaluation and quantification of the amplified target region after the reaction has been stopped, which is why semiquantitative PCR is also sometimes known as endpoint read qPCR. Most commonly, this is accomplished by running a sample of the reaction stained with an intercalating agent such as ethidium bromide on an agarose gel and then comparing band intensities. Other used post-PCR quantification methods involve adding labeled cDNA probes, incorporating radiolabel into the reaction, or running the reaction through HPLC columns. Although there are many different methods of using semiquantitative PCR methods in the quantification of RNA and DNA, these techniques are basically variations of noncompetitive and competitive qPCR reaction methods.

Noncompetitive qPCR

Noncompetitive qPCR does not use a competitive internal standard run in the same reaction where the sequence of interest is being quantified. This method relies on the observation, now well established, that prior to the onset of the plateau effect

there is a linear relationship between the quantity of input RNA and final product during PCR amplification. This technique may require initial studies to determine the number of cycles at which the linear relationship occurs for each specific primer set and at what concentration. Alternatively, the sample can be diluted to extinction and the dilutions are then amplified in separate reactions or multiple reactions can be run for each sample and stopped at different cycle end points for evaluation to determine when the reaction begins to become nonlinear. Then with knowledge of the amplification efficiency and end product level, the starting template can be calculated. Initially, there were concerns regarding the potential that a deficiency in priming could occur as the mRNA sequence of interest is diluted in more concentrated total RNA, thus affecting cDNA concentrations in the PCR. Therefore, though two samples start out with the same copy number for an mRNA of interest if one sample is diluted in a higher concentration of total RNA, reduction in priming might potentially lead to reduced amplification of the mRNA of interest from that sample. This has been tested by diluting total RNA from tissue expressing high copy numbers of a specific gene in total RNA from tissue that expresses negligible amounts of the specific gene of interest so that the quantity and concentration of RNA remained the same, but the amount of RNA from tissue that expressed high copy numbers decreased by 50% with each dilution. Results showed that a linear relationship between input RNA and final RT-PCR product was maintained throughout the dilution range (Halford et al. 1999).

Competitive qPCR

The competitive qPCR assay is usually performed by titrating a known quantity of an internal standard target against a constant amount of the experimental mRNA or DNA target in a single reaction. The concentration at which the product from the internal standard target equals the product produced from the experimental target is taken to be the starting concentration of the experimental sequence of interest. These internal standards are also called "mimics," as the standard gene is supposed to have very similar characteristics with the experimental gene so that the amplification efficiencies are identical. Given the potential differences between PCR mimics and the experimental target, particularly with heterologous standards, it is essential to determine empirically whether the target and mimic sequences amplify with similar efficiencies. To do this, one can plot the log of the product against the cycle number for the experimental and the standard target. The similarity of the slopes for the linear portion of the resulting two curves is indicative of the similarity of the amplification efficiency. Successful PCR mimics for a large number of genes have been developed and are commercially available (Siebert and Larrick 1993; Illmer et al. 1999). The internal standard necessarily differs from the target sequence in relation to fragment size or sequence so that the amplified fragments can be discerned from each other. There may be, for example, a deletion, an insertion, or a mutation such as a unique restriction site in the standard sequence. Thus, the two PCR products can be distinguished by gel electrophoresis according to size, by hybridization, or through restriction fragment analysis. Optimally, the internal standard resembles the target as much as possible, since the amplification efficiency is related to the length and sequence of the fragments. It has been suggested that the nature of competitive PCR

makes it possible to obtain useful data after the reaction has reached the plateau phase (Stalbom et al. 1994; Takara et al. 2003). This would be a considerable advantage, because quantitation could be obtained postreaction by simply performing agarose gel electrophoresis of ethidium bromide-stained PCR products without assessment for ending the reaction prior to the consumption of reaction components and product degradation. However, most researchers caution that the competitive PCR technique has substantially reduced accuracy when the product is measured after the plateau phase (Freeman et al. 1999; Weiss and Albermann 2003), particularly so when similar primers are used for amplification of an unknown and differentially sized competitive standard.

Real-Time qPCR

Since the late 1990s, RT-qPCR has rapidly been replacing semiquantitative PCR in most laboratories and is currently the most widely accepted method for the determination of absolute or relative copy number in gene expression and microarray validation. The technology to detect PCR products in real time has been available for more than 20 years, but in the past few years it has seen a dramatic increase in use. A MEDLINE search using real time and PCR as key words yielded 52 citations in 1998, 3,522 citations in 2003, and more than 4,800 citations in only the first 6 months of 2008. The enhanced sensitivity, increased range of detection, and potential for higher throughput of RT-PCR, as compared to regular PCR or semiquantitative PCR and other detection methods, have also led to an increase in its use for simple sample sequence detection (Powell et al. 2005; Baric et al. 2006).

Probably, the most significant use of RT-qPCR involves measuring expression patterns and comparing mRNA levels from different samples. In the cells of all living organisms, specific cellular activity is regulated by modulation of gene expression. Gene expression plays a significant role in determining the copy number of mRNA that exists for a particular gene. In some studies, changes in mRNA expression have been shown to correlate directly with protein level and/or activity, and are being evaluated for physical traits (Rodrigues et al. 2006; Chen and Regan 2007; Huang and Pan 2007).

Real-time techniques integrate the amplification and analysis steps of the qPCR by monitoring the amplification of DNA product of each PCR cycle. Although qPCR has been around since the late 1980s, the technology of RT-PCR was originally developed only a decade ago, around 1996 (Clementi et al. 1995; Heid et al. 1996). The first commercially available instrument, the LightCycler, was built and distributed by Idaho Technology (Idaho Falls, ID, USA) and was later purchased by Roche (Basel, Switzerland). The development of several practical real-time fluorescence-based qPCR systems since 2000 has also enhanced the impetus for this technology. As is shown in Table 3.1, several companies currently manufacture RT-PCR fluorescent detection machines and several different methods have been derived for the utilization of fluorescent dyes to monitor product amplification real time. The simplest method uses fluorescent dyes, mainly SYBR Green (Invitrogen, Carlsbad, CA, USA), that bind specifically to double-stranded DNA. Otherwise, fluorescent-labeled probes such as molecular beacons, Scorpions, and Taqman are used that bind site specifically to amplicons.

Table 3.1. Example of real-time analysis companies and their products.

Example of real-time analysis machines	Company
LightCycler Systems	Roche Applied Science, Basel, Switzerland
Biometra	Labrepco, Horsham, PA, USA
Cepheid Smartcycler	Cepheid, Sunnyvale, CA, USA
Mx4000	Stratagene, LaJolla, CA, USA
iCycler, CFX96	Bio-Rad, Hercules, CA, USA
ABI 5000, 7900	Applied Biosystems, Foster City, CA, USA
Rotor-Gene	Corbett Life Science, Sydney, Australia
ep realplex	Eppendorf, Hamburg, Germany

Fluorogenic labeling, a complementary sequence oligonucleotide, usually the probe, is currently the most extensively used method for most RT-PCR applications. Prior to fluorescent labeling of amplified products, radioactive labeling was consistently used for its high sensitivity, but fluorescence has since replaced it because most researchers found it easier than contending with the short half-life of labeled probes and the disposal and handling of hazardous radioactive material. The incorporation of fluorescent labels for detection has tremendously expanded the utilization of qPCR in research studies by increasing the sensitivity and reproducibility of qPCR reactions, along with significantly reducing the time between sample isolation and final results. Unlike semiquantitative PCR, the use of RT-PCR limits the extensive optimization that was necessary with early qPCRs. The level of amplified products is now measured after every cycle, reaction efficiency is directly determined, and the results are downloaded and directly analyzed by computer in "real time." RT-qPCR assays are also significantly less variable than semiquantitative PCR protocols which are subject to significant error. Where measured, the coefficient of variation for C_T (cycle threshold) data has been shown to be very low, between 0.4 and 2% for today's automated real-time systems versus 14% found when using conventional semiquantitative methods (Bustin 2002). The increased speed of RT-PCR over semiquantitative PCR is mainly due to the elimination of handling and measuring amplified sequence post-PCR reaction. Furthermore, RT-qPCR is performed in a closed system with the reaction run and analyzed in sealed plates. Advantages with this system include improved run reaction times, and better quality control with minimal contamination risks. It has been suggested that the reduction in amplicon size found in RT-qPCR may increase the reaction speed and PCR efficiency, but this has not actually been proven (Bustin 2000; Reid et al. 2002). Nevertheless, the use of fluorescent labels has been shown to increase sensitivity and accuracy in amplicon detection over previous and other existing methods.

Originally, the disadvantages of using RT-PCR in comparison with quantification by conventional PCR included the inability to monitor amplicon size without further analysis, the incompatibility of some platforms with certain fluorogenic chemistries, and the relatively restricted multiplex capabilities of current applications. Also, the start-up expense of RT-PCR was prohibitive when used in low-throughput laboratories. With improvements to software analysis programs and more economic hardware now available, these shortcomings are a thing of the past.

Overall, there are two basic systems for RT-PCR: probe-based and intercalator-based systems. Both methods require a modified thermocycler that is equipped with a camera for monitoring the fluorescence in each well of a plate during every cycle of the PCR. Probe-based RT-qPCR typically requires PCR primers and fluorogenic probes. The oligonucleotide probes may contain both a reporter fluorescent dye and a quencher dye on the same oligonucleotide or separately. The intercalator-based method, commonly referred to as the SYBR Green (Invitrogen, Carlsbad, CA, USA) method because this is the most prevalent reagent, requires a dye in the PCR that binds to newly synthesized double-stranded DNA and then fluoresces. Probe-based RT-qPCR has been proven to be more specific and reliable than SYBR Green method in some cases, but it is more expensive (Bustin 2005; Donia et al. 2005). Next is a description of these different chemistries and their function in RT-qPCR.

SYBR Green is an intercalating agent similar to ethidium bromide. Measuring fluorescence from DNA intercalating agents is the simplest and least expensive format for RT-PCR product detection. SYBR Green binds double-stranded DNA and upon excitation emits light. Thus, as PCR product accumulates, fluorescence increases (Figure 3.2a). Since the dye binds to double-stranded DNA, only amplification primers are needed and there is no need to design a probe for any particular target being amplified. SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction products, which can result in an overestimation of the target concentration. Therefore, detection by SYBR Green requires extensive optimization since the dye cannot distinguish between specific and nonspecific products accumulated during PCR. To verify whether only one product is being amplified, a melting curve can be generated following a reaction. The melting curve should have only a single peak as additional peaks indicate the presence of nonspecific priming or possible primer-dimer formations contributing to the fluorescent signal. Follow-up assays are usually needed to validate results. For single PCR product reactions with well-designed primers, SYBR Green can function very efficiently, with spurious nonspecific background only showing up in very late cycles. But the real advantage for SYBR Green is its flexibility allowing the user to quickly and economically analyze or validate several genes without having to purchase and evaluate fluorescent-labeled sequence-specific probes.

Fluorescent Detection Systems

Today, the vast majority of the systems sold and used in research laboratories use fluorescent detection and rely on fluorescence resonance energy transfer (FRET) (Chen et al. 1997). FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule, and thereby blocking changes in the detectable fluorescent emissions. The concept behind FRET in most quantitative RT-PCR assays is that when two reporter molecules are in close proximity, energy from the high-emitting dye is being quenched by the low-energy dye, but when these molecules become separated the high-energy dye becomes excited and releases energy that is detected and quantified by a specific detection system (Forster 1948; Van Rheenen et al. 2004).

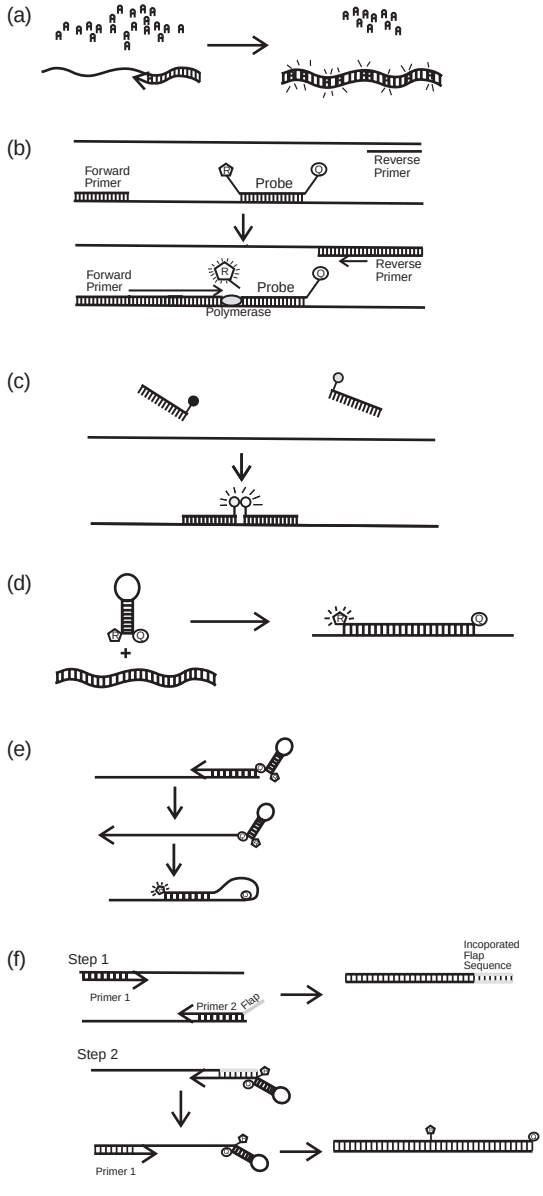

Figure 3.2. Different chemistries and probes used in quantitative RT-PCR. (a) SYBR Green, (b) 5′ nuclease or Taqman probes, (c) adjacent oligonucleotide probes or HybProbes, (d) hairpin probes or molecular beacons, (e) Scorpion probes, and (f) Amplifluor probes. The properties for each of the listed reagents are described in the text.

Most commercial RT-qPCR assays using labeled probes also include a reference dye that does not participate in the reaction but is used as an internal reference to normalize reporter dye signal. While not participating in FRET-released fluorescence reaction, the purpose for this dye is to correct for changes in concentration or volume between reactions.

5′ Nuclease or Taqman Assay

This assay makes use of the DNA polymerase that is functioning to elongate the primed sequence during the reaction and utilizes the 5′-nuclease activity of that DNA polymerase to hydrolyze an oligonucleotide probe (sometimes referred as hydrolysis probes) that is hybridized to the target amplicon (Figure 3.2b). These oligonucleotide probes have a fluorescent reporter dye attached to their 5′ end and a quencher moiety coupled to the 3′ end. The probes are designed to hybridize to an internal region of a PCR product. When the probe is not hybridized to the amplicon and intact, the proximity of the fluorophore and the quencher molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a probe is bound, the 5′-nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of cleaved probe product (Lee et al. 1993). There are many types of software that can be used to design these probes or companies such as Applied Biosystems (Foster City, CA, USA) have a service, Assay-by-Design, where given sequence they will design probes and primers for RT-PCR assays. Well-designed probes for the 5′-nuclease assay require very little optimization. In addition, they can be used for multiplex assays by designing each probe with unique fluorophore and quencher pairs (Bernard et al. 1998). However, compared to SYBR Green, these probes are relatively expensive and a separate probe is needed for each mRNA target being analyzed.

Complementary probes can be designed to cover most sequences that do not contain an overabundant ratio of cytosines and/or guanosines within the desired area. Originally, for the 5′-nuclease assay these probes were generically referred to as Taqman probes, were approximately 15–20 bp in length, and bound to the major groove of cDNA. More recently, a new type of probe termed a minor groove binding (MGB) probe has been developed, which binds to the minor groove of DNA with higher affinity than previous oligonucleotide probes (Kutyavin et al. 2000). When MGBs are conjugated with oligos, these probes form very stable hybrids with cDNA. Commercial MGB probes typically have the MGB peptide moiety placed at the 3′ end since these are easier to synthesize, but the MGB can be placed at either the 3′ or 5′ end. These probes use a nonfluorescent quencher that replaces TAMRA (6-caroxytetramethyl-rhodamine), which was used as a quencher in earlier Taqman probes. Because of their shorter length, fluorescence quenching is more efficient providing increased sensitivity, and the tighter binding endows the probes with higher melting temperatures and allows for the design of significantly shorter probes that are also more sequence specific. The shorter length and specific binding of these probes do make for added restrictions as to where they can bind, thus making it more difficult to find optimal sites for engineering a probe within a sequence of interest. These probes are consistently used in available commercial RT-PCR machines.

Adjacent Oligonucleotide Probes or HybProbes

This method requires the use of two sequence-specific primers and two sequence-specific fluorescent-labeled oligonucleotide probes. Unlike the 5′-nuclease assay, in this system, it is the binding of a pair of adjacent, fluorogenic hybridization oligoprobes

instead of just a single probe binding to the amplicon (Figure 3.2c). These probes have been predominantly used with LightCycler instruments and are also known as HybProbes. The detection principle for these types of probes again relies on FRET but uses blue light for the excitation of the donor fluorophore so that when the donor and acceptor fluorophore are in close proximity, this results in a shift in the emission to light with a longer wavelength. The upstream oligoprobe is labeled with a 3′-donor fluorophore such as FITC, and the downstream probe is commonly labeled with an acceptor fluorophore such as Red 705 at the 5′ terminus so that when both oligoprobes are hybridized to their complementary sequence, the two fluorophores are located within a few base pairs of each other. These types of probes have also been termed "kissing probes," because fluorescent energy is measured when the two probes are close together. In the case of this assay when the two fluorophores on the different probes are brought within close proximity to each other, a detectable signal at a specific wavelength results, which is then measured for quantitative analysis. Then when the temperature of the reaction is increased above the melting temperature for the oligonucleotide probes, the probes disengage from the template strand, thereby increasing the distance between the fluorophores and the signal rapidly diminishes as the oligonucleotides move farther apart.

Hairpin Probes

Molecular beacons (Tyagi and Kramer 1996), Scorpions (Whitcombe et al. 1999), and Amplifluor primers (Nazarenko et al. 1997) are oligonucleotide probe/primer constructs that use a hairpin structure that places the donor and acceptor moieties in close proximity to maximize the quenching effect. The quencher is typically a nonfluorescent aromatic moiety such as 4-((4-(dimethylamino)-phenyl)-azo)-benzoic acid (DABCYL) or a heterocycle compound that masks the fluorescent properties of the fluorophore due to close contact (Yaron et al. 1979). Molecular beacon probes bind to an amplicon generated by two separate primers (Figure 3.2d), much like Taqman probes. The probe contains both an acceptor and donor fluorophore on one oligonucleotide. Prior to binding to the amplicon the ends of the oligonucleotide are twisted in a hairpin loop, bringing the two fluorophores in close contact and quenching the signal. Upon binding to the complementary sequence of the amplicon, the labeled probe is straightened, putting sufficient distance between the ends, and fluorescent energy is released.

Scorpion Probes

With Scorpion probes the fluorophore and quencher are both part of a single primer. As with molecular beacons, the primer contains a hairpin loop that brings the donor and acceptor fluorophore in close proximity. After the primer binds and the polymerase extends the primer sequence, the hairpin section of the primer then comes undone, loops out, and complementarily binds to the new sequence, whereby the fluorophores are separated and a signal is released (Figure 3.2e). Amplifluor uses a two-step system, whereby one of the primers used during PCR amplification has

additional noncomplementary bases attached that allows an extra sequence to become incorporated into the amplicon. Then during the second step, the hairpin probe binds to the primer incorporated sequence and during incorporation of the attached probe into the double-stranded product, the hairpin structure becomes unfolded, separating the fluorophore and quencher, and produces a signal that then correlates with the amount of amplification product (Figure 3.2f).

Other Methods

DzyNA-PCR is another similar method that uses a primer with a hairpin loop that contains a fluorophore and quencher within close proximity. Successful amplification of target DNA, and concomitant cleavage of the reporter substrate by amplicons containing active DNAzymes produces an increase in fluorescent emission of the fluorescent reporter (Todd et al. 2000).

Other real-time techniques that should be mentioned include the Invader assay and the use of peptide nucleic acid (PNA) oligomers. The Invader assay is designed to use two synthetic oligonucleotides that hybridize to the sequence of interest in a partially overlapping manner, leaving a flap of the oligonucleotide unbound. An endonuclease enzyme is included in the reaction and functions to cleave the overlapping flap structure. This flap structure is then involved in a secondary reaction where it serves as an invasive oligonucleotide in a second cleavage reaction. The substrate of the second reaction is an oligonucleotide that contains the fluorophore and quencher in close proximity, whereby hybridization of the flap stimulates cleavage and a fluorescent signal (Allawi et al. 2004).

PNA oligomers are analogous to DNA oligonucleotides except the phosphate backbone of the DNA molecule is replaced with repeating *N*-(2-aminoethyl)-glycine units linked together by peptide bonds (Nielsen et al. 1991). The unique characteristic of PNA oligomers, such as not possessing ionic charge, allows for the deletion of salt in reactions for the stabilization of duplexes. This means that under low-salt conditions, PNA will bind more effectively than competing DNA strands, and PNA/DNA duplexes will have a higher T_m, allowing for the utilization of probes with shorter lengths. These probes are also more sensitive to mismatched base pairs since a lower T_m is required to bind than for DNA/DNA duplexes (Fiandaca et al. 2001).

While this description of techniques is not exhaustive, it does cover most of the currently used methods or provides a basic description from which other marketed techniques are modified. These described fluorescent-labeled probes allow for the multiplexing of analysis, whereby multiple DNA samples may be measured in the same well. This is possible because fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be coamplified and permits discrimination of expression between multiple genes in a single tube. Hybridization probes afford a level of discrimination impossible to obtain with SYBR Green, since they will only hybridize specifically to complementary sequences in a PCR and not to primer-dimers or other spurious products. Also because of the sensitivity of these fluorescent-labeled probes to detect even a single base pair mismatch, they have become useful for the detection of allelic discrimination and evaluation of specific point mutations (Walker 2001; Burgos et al. 2005).

Calculation and Standardization Methods

Copy numbers of DNA or cDNA are determined during the exponential phase of the reaction by plotting fluorescence against cycle number on a logarithmic scale. The point of amplification where detection of fluorescence becomes greater than background fluorescence is the C_T for detection, and this point is typically used for calculating relative or absolute copy number. It should be noted that C_T values are inversely related to the cycle number and determined amount of amplified DNA product (Giulietti et al. 2001). During the amplification phase of the reaction, the quantity of DNA is doubling every cycle, which allows for the determination of copy numbers. For example, a sample whose C_T value is 15 after 20 cycles of amplification will have 16 times more copies than a sample whose C_T value is 15 after 24 cycles ($2^4 = 16$).

There have been several different methods described in the literature for transforming and presenting the data obtained from qPCR reactions. When comparing between experimental and control samples, data can be reported as raw values normalized to housekeeping genes (Overturf and LaPatra 2006; Johansen et al. 2006), fold change in expression (Purcell et al. 2004), or changes in C_T values (Olsvik et al. 2005). Most results are reported as relative values as opposed to expression of absolute copy numbers. As noted before, when absolute copy number is expressed, the copy number of the sample is determined from serially diluted standards of known copy number values. This is then standardized to total RNA or expression of a housekeeping gene. For relative expression, a number of methods have been determined as adequate for analysis and presentation of the data. One method reported by Pfaffl (2001) is calculated using the reaction efficiency and the threshold deviation of an unknown sample against a control. This method gives the relative expression ratio of a gene of interest versus a control in comparison to a reference gene. The ratio is calculated using the following equation:

$$\text{Ratio} = \frac{(\text{PCR efficiency of gene of interest})^{\Delta C_T(\text{Control } C_T - \text{sample } C_T)}}{(\text{PCR efficiency of reference gene})^{\Delta C_T(\text{Control } C_T - \text{reference } C_T)}}$$

The comparative C_T method or ${}^{\Delta\Delta}C_T$ method are similar methods to calculate relative differences between experimental and control samples (Livak and Schmittgen 2001). This method does not require standard curves to be run with each plate or set of reactions and is functional for estimations of relative expression ratios. For this method the efficiency of amplification needs to be approximately equal for the reference and target sequences. Unequal efficiencies between the target and reference sequence will proportionally diminish the accuracy in calculated fold change of expression (Peirson et al. 2003).

Many researchers have reported relative differences in expression simply by referencing the normalized data to a relative standard curve. This method uses a set of relative standards to which unknown samples are then quantified. The standard curve can consist of using serial dilutions of an arbitrarily quantified control sample. Quantification of test samples is then performed by comparative analysis with the standard curve. The data are then normalized to RNA concentration or an expressed control gene for which a serial standard should also be run on the same plate. As with other

methods, good reaction efficiencies are required and R^2 values for standard curves should be greater than 95%.

Researchers also typically normalize the expression of a gene of interest to what is referred to as a housekeeping gene. These are genes that are thought to be constitutively expressed in most cell types at relatively equivalent levels per tissue. In regards to control or housekeeping genes, there consists of a vast list of genes that have been reported as being stably expressed in specific tissues (Radoni et al. 2003). It should be noted that the use of a specific gene for normalization hinges upon the fact that experimental treatment will not impact expression of the control gene versus untreated samples. Small et al. (2008) demonstrate changes in several currently used control genes in different tissues from catfish (*Ictalurus punctatus*) under varied experimental conditions. Elsewhere, Olsvik performed similar experiments evaluating six housekeeping genes in Atlantic salmon (Olsvik et al. 2005) and recently the expression of ten reference genes were looked at in multiple tissues for the three-spined stickleback, *Gasterosteus aculeatus* (Hibbeler et al. 2008). Many of these genes have been evaluated elsewhere (Thellin et al. 1999; Ke et al. 2000; Jorgensen et al. 2006). A list and description of several genes used for controls are given in Table 3.2.

qPCR in Aquaculture Research

qPCR has been widely used in aquaculture studies. Although some semiquantitative PCR work is still being done, most of the studies, now reported and accepted, utilize RT-qPCR for analysis. There are several available quantitative assays developed and available for a number of pathogens including bacteria, viral, microsporidians, and others. A sample list includes *Renibacterium salmoninarum* (bacterial kidney disease), *Myxobolus cerebralis* (whirling disease), infectious hematopoietic necrosis virus, and *Nucleospora salmonis* (Overturf et al. 2001; Cavender et al. 2004; Powell et al. 2005; Foltz et al. 2009). Certain groups have developed RT-PCR assays for pathogens and are using them qualitatively instead of quantitatively, as a more sensitive method for the detection of pathogens. Yet other designed assays have gained utility currently as a method for calibrating the efficacy of existing pathogen quantitative techniques with the goal being to replace these techniques with a more sensitive, economical, and faster RT-qPCR assay. Many studies have also used qPCR to monitor the presence of a pathogen in processed products (LaPatra et al. 2001).

As these studies demonstrate qPCR has proven extremely useful for the detection and quantification of pathogens. However, most of the studies reported by aquaculture show researchers using this technique in comparing the relative expression of genes. Research publications in aquaculture show qPCR being used in a number of studies evaluating expression changes in areas related to disease, growth, and other physiological parameters. Because of the huge economic impact that disease has on aquaculture production, immunological studies evaluated the expression of these genes in several different fish species including salmonids, flounder, catfish, carp, and others (Malina et al. 2004; Peterson et al. 2005; Johansen et al. 2006; Overturf and LaPatra 2006; Yasuike et al. 2006). Most of the studies look to determine what specific immunological genes and/or pathways are upregulated upon pathogen exposure. In some studies, changes in immunological gene expression have been linked to genetic variability with disease resistance (Bilodeau-Bourgeois et al. 2008), while in other studies correlations

Table 3.2. List of control genes used for standardization of real-time qPCR reaction.

Gene	Description	References
β-actin	A highly conserved nonmuscle cytoskeleton protein. Highly expressed in all vertebrate cell types	Sarraf et al. (1998) and Kreuzer et al. (1999).
Albumin	Globular protein used in transport of compounds throughout the body. Expression of this gene has been found	Goldsworthy et al. (1993) andMarten et al. (1994)
18S ribosomal RNA	Subunit of eukaryotic ribosomes involved in the initiation of polypeptide synthesis. This is a highly expressed gene that is actively transcribed in all cell types	Jorgensen et al. (2006) and Small et al. (2008)
GDPDH	Glyceraldehyde phosphate dehydrogenase is one of the initial reference genes used in the standardization of Northern blots and then qPCR. It is involved in the breakdown of glucose and also several other nonmetabolic processes. Its expression has been found to vary in different tissues and under variable physiological conditions	Tang et al. (1996) and Foss et al. (1998)
HPRT	*Hypoxanthinephophoribosyltransferase* is a constitutively expressed gene involved in the salvaging and recycling of nucleotides. Its expression level is relatively low compared to others, but its expression is found to vary especially in growing cells	Marten et al. (1994) and Foss et al. (1998)
EF1A	Elongation factor 1A is a ubiquitous expressed protein that elongates polypeptides during translation. Its expression has been found to vary in different tissues and under different physiological conditions	Edmonds et al. (1996) and Welle et al. (1997)
G6PDH	Glucose-6 phosphate dehydrogenase is a rate-limiting enzyme in the pentose phosphate pathway. Although ubiquitously expressed with multiple isoforms, its expression has been found to vary between tissues and under different conditions	Ninfali et al. (1995) and Gao et al. (2004)
Alpha tubulin	Alpha tubulin is globular protein involved in the makeup of microtubules and is ubiquitously expressed in tissues	Bogaert et al. (2006)

in immunological gene expression in response to pathogen have been delineated (Overturf and LaPatra 2006). This research has enhanced our understanding of the cellular and humoral components in different fish species after infection with diverse pathogenic organisms (Huising et al. 2006; Matsuyama et al. 2006; Saint-Jean and Perez-Prieto 2007). Expression of immunological genes has also been used to study the effect of probiotics and other immunostimulants on the immune system when fed these products (Low et al. 2003; Sealey et al. 2007a, 2007b).

qPCR is also being used in nutritional studies in fish. Originally, diets for most species were formulated by manipulating diets already in use for other species. Now with the formulation of diets specific for growth stage such as larval, grow-out, and brood, and for species, research has begun to determine how to incorporate the best nutrition into an economically feasible diet. For example, in salmonid culture, the goal is to limit fish meal and fish oil in diets as much as possible and to replace with sustainable plant protein. RT-PCR was used to evaluate and demonstrate changes in metabolic enzymes after an amino acid modification of an all-plant diet (Gaylord et al. 2007). Carbohydrate utilization in fish has shown alterations in the expression of specific glycolytic enzymes according to species and level (Panserat et al. 2001, 2002). Some of this work is presented in more detail in Chapter 10.

qPCR analysis is being used to evaluate gene expression activity in fish during different stages of development between fish displaying differential growth. This includes research on growth hormones and their receptors in different species (Small and Peterson 2005; Mao et al. 2007) and studies involving transgenically modified species (Mori and Devlin 1999; Morales et al. 2001; Rahman et al. 2004). Other studies are looking to determine precisely what roles hormones and other proteins of the endocrine system have in muscle differentiation and development (Biga et al. 2004; Johansen and Overturf 2005, 2006). These studies have aided in defining the genes involved in muscle regulation and how they interact and are regulated in fish according to age and growth rate. Related research is involved in examining the expression of protein degradation and synthesis of genes and how modulation of these pathways translates in protein retention and growth (Salem et al. 2005, 2006). From these studies, it appears that while in mammals ubiquitin labeling and digestion in the proteasome is the major form of degradation, in fish the cathepsin and calpain pathways look to be the major forms of degradation in muscle turnover. In salmonids, two forms of myostatin have been discovered. One form is found to be highly expressed in muscle and its increased expression is found to correlate with a decrease in muscle development, similar as to what has been found in terrestrial mammals. However, unlike in mammals, another myostatin gene called *myostatin II* is also expressed and qPCR shows its expression is higher in tissues other than muscle and appears to be more closely linked to metabolic regulation (Ostbye et al. 2001; Rescan et al. 2001). This is related to quantitative gene expression analysis done by others with an interest in gene identification and then correlation of its expression with physiological changes in growth (Burke et al. 1998; Chang et al. 2003; Robinson et al. 2008). These genes could have potential for use as molecular markers in genetic selection programs.

While more and more aquaculture researchers are using RT-qPCR in their laboratories for evaluating potential gene expression variance for specific genes in tissues from individuals under different experimental conditions, a significant increase in the use of RT-qPCR has occurred because of its use as a method for validation in microarray studies. With the development of more sophisticated arrays for well-studied

species and the rapid increase in sequence information leading to the generation of microarrays for newer species, more researchers have either used RT-PCR to validate the findings from their microarray experiments or benefited from sequence data generated from the EST libraries produced and analyzed for microarray development.

Future Directions and Use of qPCR

As aquaculture continues to expand with the domestication and development of new species and the continued understanding and improvement of existing important aquaculture species, RT-qPCR will play an increasing role in correlating gene expression with physiological traits. Because qPCR is so sensitive and rapid and can analyze hundreds of samples, with replicates, in a single run, it will continue to be used as a technological tool for probing studies to determine if changes in specific genes of interest are related to physiological changes or as a more sensitive method of detection of change. As the information regarding expression levels of specific genes and their correlation with physical effects becomes known, it seems probable that panels of possibly 10–25 genes will be developed for testing for specific traits in different strains and/or species of fish.

The comparative ease with which RT-PCR assays can generate quantitative data has created the impression that this technique can be run directly "off the shelf" and that data can be subjected to objective statistical analysis. However, normalization procedures between samples must be properly validated to achieve biologically relevant interpretation of data. Experimental protocols and designs need to be rigorously controlled and allow meaningful global comparisons between research aquaculturists. This information then needs to be disseminated and discussed among researchers to piece together how transcriptional regulation of specific groups of genes plays a role in affecting animal physiology.

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Chapter 4
Aquaculture-Related Applications of DNA Microarray Technology

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Introduction

DNA microarrays printed on glass slides have been available for some aquaculture-relevant species since 2004, and global gene expression studies involving new aquatic animal DNA microarray platforms are appearing in the literature virtually every month. In a very short time, DNA microarrays have become popular tools for aquaculture-related research. This is not surprising given that these genomic tools have recently revolutionized scientific research in other areas such as toxicology, agriculture, biomedicine, and developmental biology. There is a growing literature on the use of fish DNA microarrays for studies related to toxicology (e.g., Finne et al. 2007; Gunnarsson et al. 2007) and biomedicine (e.g., Meijer et al. 2005; Lam et al. 2006) that is beyond the scope of this chapter. We will focus on microarray-based research that is directly relevant to current aquaculture industry concerns (e.g., toward developing immune-robust and rapidly growing broodstock).

Aquaculture genomic research aims to develop a thorough understanding of the genes and molecular pathways involved in biological processes important for the optimal culture of aquatic animals, in particular growth, reproduction, immune responses, and responses to stress. Microarray experiments building on appropriate experimental paradigms and utilizing appropriate microarray platforms can identify gene expression correlates for production-relevant traits of interest to the global aquaculture industry (e.g., resistance to pathogens or environmental stress). This work may lead to the development of new molecular tools (markers) and techniques for identifying genetically superior broodstock.

In this chapter, examples from the literature are provided on how DNA microarrays have been used to improve our understanding of the molecular bases of physiological processes that are important in aquaculture research (e.g., fish responses to pathogens, vaccines, and environmental stresses). Due to their prevalence in the literature and prominence as aquaculture species, we focus the introduction on the results of microarray-based research on salmonids, catfish, and flatfish (Table 4.1). Examples of microarray studies on other aquaculture finfish such as carp (e.g., Gracey et al. 2004) and sea bream (e.g., Sarropoulou et al. 2005) are mentioned in Table 4.1, but are not expounded upon in the text. In addition, a large-scale genomic research project on Atlantic cod (*Gadus morhua*) has generated high-complexity cDNA libraries and expressed sequence tags (ESTs) (e.g., Rise et al. 2008), with a DNA microarray

Table 4.1. Examples of publications using fish DNA microarrays for aquaculture-relevant research.[1]

Species studied	Reference	Microarray platform[2]	Application
Atlantic salmon (ESTs: 433,337)	Rise et al. (2004a)	GRASP 3.5K cDNA	Gene expression response (GER) to *P. salmonis*
	Jordal et al. (2005)	73 gene lipid metabolism	Influence of diet on hepatic gene expression
	Ewart et al. (2005)	IMB 4K cDNA	Immune tissue GER to *Aeromonas salmonicida*
	von Schalburg et al. (2005b)	GRASP 16K cDNA	Cross-species (heterologous) hybridizations
	Martin et al. (2006)	GRASP 3.5K cDNA	GER to live *Aeromonas salmonicida* vaccine
	Morrison et al. (2006)	GRASP 16K cDNA	Gill GER to amoebic gill disease parasite
	Roberge et al. (2007)	GRASP 16K cDNA	Whole body GER to fungal disease
	Ewart et al. (2008)	IMB 4K cDNA	Macrophage GER to *Aeromonas salmonicida*
	Young et al. (2008)	GRASP 16K cDNA	Study of gene expression changes in AGD lesions
	Wynne et al. (2008)	TRAITS 17K cDNA	GER in AGD-affected tissues (gill, kidney, and liver)
	Jørgensen et al. (2008)	1.8K salmonid cDNA	GER to infectious salmon anemia virus (ISAV)
Rainbow trout (260,887)	Rise et al. (2004b)	GRASP 3.5K cDNA	Effectiveness of cross-species hybridizations
	Sneddon et al. (2005)	Rainbow trout brain	Gene expression correlates of dominance status
	Krasnov et al. (2005a)	Kuopio 1.3K cDNA	Identification of stress-responsive genes
	von Schalburg et al. (2005a)	GRASP 3.5K cDNA	Gene expression profiling of developing ovary
	Vornanen et al. (2005)	Kuopio 1.3K cDNA	Heart GER to cold acclimation
	MacKenzie et al. (2006a)	Kuopio 1.4K cDNA	GER of cultured macrophages to LPS and cortisol
	Salem et al. (2006)	GRASP 16K cDNA	Muscle GER to atrophy during vitellogenesis
	Johansen et al. (2006)	GRASP 16K cDNA	Muscle and liver GER to chronic LPS treatment
	Purcell et al. (2006)	GRASP 16K cDNA	Muscle GER to IHNV DNA vaccine injection
	Kirchner et al. (2007)	GRASP 16K cDNA	Gut GER to different levels of dietary phosphorus
	Martin et al. (2007)	GRASP 16K cDNA	Macrophage GER to recombinant cytokines

	Bonnet et al. (2007)	INRA 9K cDNA	Egg gene expression in natural versus controlled ovulation
	Gerwick et al. (2007)	OSU 1.6K oligonucleotide (oligo)	Liver GER to killed bacteria in adjuvant
	Salem et al. (2007)	GRASP 16K cDNA	Liver GER to starvation
	von Schalburg et al. (2008b)	GRASP 16K cDNA	Gene expression changes during gonadogenesis
	Olohan et al. (2008)	21.5K 60-mer oligo	Cultured trout fibroblast cell GER to anoxia
	Salem et al. (2008)	37K 60-mer oligo	Muscle GER to vitellogenesis-induced atrophy
Coho salmon (2,325)	Rise et al. (2006)	GRASP 3.5K, 16K, and IMB 4K	Liver GER to growth hormone (GH) transgenesis ± rationing (cross-species hybridizations)
Amago salmon (21)	Mori et al. (2007)	0.5K Amago cDNA	Liver GER to GH transgenesis
Channel catfish (44,767)	Ju et al. (2002)	660 cDNA nylon	Brain GER to cold acclimation
	Li and Waldbieser (2006)	19K in situ oligo	Spleen GER to lipopolysaccharide (LPS)
	Peatman et al. (2007)	28K in situ oligo	Channel catfish liver GER to bacterial pathogen
Blue catfish (10,764)	Peatman et al. 2008	28K in situ oligo	Blue catfish liver GER to bacterial pathogen *Edwardsiella ictaluri*
Atlantic halibut (18,495)	Douglas et al. (2008)	9279 gene Atlantic halibut oligo	Differential gene expressed during larval development (hatching to postmetamorphosis)
Japanese flounder (8,822)	Kurobe et al. (2005)	871 gene cDNA	Kidney cell GER to immunogenic stimuli
	Byon et al. (2006)	1187 gene cDNA	Head kidney GER to different vaccine types
	Matsuyama et al. (2007)	1187 gene cDNA	Leucocyte GER to *E. tarda* vaccine challenge

(*continued*)

Table 4.1. (*Continued*)

Species studied	Reference	Microarray platform[2]	Application
Zebrafish (1,379,829)	Malek et al. (2004)	16K zebrafish oligo	Muscle GER to long-term temperature reduction
	Rawls et al. (2004)	16K zebrafish oligo	Gut GER to normal (nonpathogenic) microbes
	van der Meer et al. (2005)	16K zebrafish oligo	Gill GER to hypoxia
	Harden et al. (2006)	16K zebrafish oligo	Olfactory epithelium GER to an artificial odorant
	Santos et al. (2007)	17K zebrafish oligo	Comparison of male and female gonad gene expression
Common carp (32,046)	Gracey et al. (2004)	13.4K common carp cDNA[3]	Identification of cold-responsive genes in several tissues (e.g., gill, kidney, brain, heart, and muscle)
Sea bream (32,363)	Sarropoulou et al. (2005)	10K gilthead sea bream cDNA	Gene expression profiling during embryogenesis and identification of stress-responsive genes

[1]This list is not intended to be exhaustive, but rather to provide examples of how finfish DNA microarray platforms and associated aquaculture-related applications have developed over approximately the past 6 years.
[2]Information on fabrication and additional applications of the GRASP 3.5K and 16K microarrays (primarily Atlantic salmon probes), the IMB 4K microarray (Atlantic salmon probes), the Kuopio microarrays (rainbow trout probes), and the Oregon State University (OSU) 1.6K oligo microarray (rainbow trout probes) may be found in Rise et al. (2007). The 17K TRAITS (TRanscriptome Analysis of Important Traits of Salmon) Atlantic salmon cDNA, 21.5K rainbow trout oligo, and 37K rainbow trout oligo microarrays are described in Taggart et al. (2008), Olohan et al. (2008), and Salem et al. (2008), respectively. K: 1,000 genes. Numbers of expressed sequence tags (ESTs) for fish species were accessed, using the Taxonomy Browser feature of the NCBI web site, on June 26, 2008. Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss*; coho salmon, *O. kisutch*; amago salmon (or cherry salmon), *O. masou*; channel catfish, *Ictalurus punctatus*; blue catfish, *I. furcatus*; Atlantic halibut, *Hippoglossus hippoglossus*; Japanese flounder (or bastard halibut), *Paralichthys olivaceus*; zebrafish, *Danio rerio*; common carp, *Cyprinus carpio*; gilthead sea bream, *Sparus auratus*.
[3]A new, 26K common carp cDNA microarray was recently described in Williams et al. (2008).

platform currently being designed and built. Microarray studies on shellfish such as oyster (Jenny et al. 2007) and shrimp (Robalino et al. 2007; de la Vega et al. 2007) may be found in the recent literature, but are not discussed in this chapter. In addition to our presentation of salmonid, catfish, and flatfish microarray experimental results, we also discuss selected zebrafish (*Danio rerio*) global gene expression studies (Table 4.1). Since this species offers excellent resources (e.g., more than 1 million ESTs, a draft genome sequence, and genetic and physical maps) and other research advantages (e.g., rapid development and transparent chorion), it may be a useful model species for fish research. Genomic resources, including more than 600,000 ESTs in GenBank and an approximately 8,000 gene (8K) oligonucleotide (oligo) microarray (Ju et al. 2007), also exist for Japanese medaka (*Oryzias latipes*). However, due to the prevalence of zebrafish microarray studies in the literature, our Introduction will focus on this species as a potential model for aquaculture research. Further information on genomic resources of model organisms is provided in Chapter 7.

In the Examples of Novel Microarray Platforms for Aquaculture-Related Research section of this chapter, we present four examples of novel microarray platforms (catfish in situ oligo, halibut oligo, fish pathogen cDNA, and host–symbiont pair of microarray platforms), and discuss their current and potential future applications in aquaculture-related research. For detailed descriptions of construction of microarray platforms for other aquaculture species, the reader is referred to the literature (Table 4.1) and to recent reviews (e.g., Douglas 2006; Rise et al. 2007). In the Future Directions section, we briefly consider the future of microarray-based tools for aquaculture research.

Aquaculture-Relevant Microarray Research on Salmonids

Salmonid DNA microarrays, first appearing in the literature in early 2004 (Rise et al. 2004b), have been used to study the genes and molecular pathways involved in various normal and pathological processes. Salmonid DNA microarray platforms include (1) an approximately 3,500 gene (3.5K) salmonid (primarily Atlantic salmon) cDNA microarray produced by the Genomic Research on Atlantic Salmon Project (GRASP) (Rise et al. 2004b), (2) a 16K salmonid (primarily Atlantic salmon) cDNA microarray produced by GRASP (von Schalburg et al. 2005b), (3) a 4K Atlantic salmon cDNA microarray produced by the Institute for Marine Biosciences (IMB) of the National Research Council of Canada (Ewart et al. 2005), (4) a 73-gene Atlantic salmon cDNA microarray designed for studies involving lipid metabolism (Jordal et al. 2005), (5) a 17K Atlantic salmon cDNA microarray produced by a European collaboration involving UK-based TRAITS (TRanscriptome Analysis of Important Traits of Salmon) and the Norwegian SGP (Salmon Genome Project) (Taggart et al. 2008), (6) an approximately 1.4K rainbow trout cDNA microarray produced at the University of Kuopio, Finland (Koskinen et al. 2004a, 2004b), (7) a 1.6K rainbow trout oligo (70 nucleotides long, or 70-mer) microarray produced at Oregon State University (OSU) (Tilton et al. 2005), (8) a 9K rainbow trout cDNA microarray (printed on nylon rather than glass) produced at the French Institut National de la Recherche Agronomique (INRA) (Bonnet et al. 2007), (9) a 147-gene rainbow trout cDNA microarray focused on genes with specific molecular functions (e.g., metabolism, stress, and immune response) (Wiseman et al. 2007), (10) a USDA-funded 37K rainbow trout 60-mer oligo microarray synthesized in situ using inkjet printing (Salem et al. 2008), (11) a 21.5K

rainbow trout 60-mer oligo microarray also built using inkjet technology (Olohan et al. 2008), and (12) an approximately 32K salmonid cDNA microarray being designed and built by GRASP (von Schalburg et al. 2008a). Numerous published studies have used salmonid DNA microarrays to study rainbow trout gene expression responses (GERs) to environmental toxicants (e.g., Koskinen et al. 2004b and Krasnov et al. 2005b, both using the Kuopio 1.4K microarray; Tilton et al. 2005, using the 1.6K OSU microarray; and Finne et al. 2007, using the GRASP 16K microarray). Aquatic organisms are emerging as important models for research using genomics tools and techniques to study how anthropogenic environmental stressors influence biological parameters relevant to animal fitness (e.g., development, behavior, and physiology) (Cossins and Crawford 2005; Denslow et al. 2007; Ju et al. 2007). Toxicogenomics research using fish and shellfish as models is relevant to aquaculture, since farmed aquatic animals may be exposed to toxicants in their feed (e.g., residual pesticides in plant-based components or bioaccumulative pollutants such as mercury and dioxins in animal-based components) or environment. Due to space constraints, we refer the reader to the toxicogenomics and environmental genomics literature and focus our discussion on microarray-based studies aimed at elucidating the genes and molecular mechanisms involved in fish immunity, growth, and reproduction.

Salmonid Immune Responses

There have been several publications involving the use of salmonid DNA microarrays to study fish global GERs to pathogens. For example, the 4K IMB microarray and standard reverse transcription-polymerase chain reaction (RT-PCR) were used to identify Atlantic salmon liver, spleen, and head kidney genes responsive to the bacterial pathogen *Aeromonas salmonicida* (Ewart et al. 2005). The GRASP 3.5K microarray was used to identify Atlantic salmon macrophage and hematopoietic kidney (head kidney) genes responsive to the intracellular bacterial pathogen *Piscirickettsia salmonis* (Rise et al. 2004a). For each of these studies, the host GER to pathogen was evaluated at a single time point in the pathogenic process (e.g., 13 days after onset of cohabitation with infected fish in Ewart et al. (2005), and 14 days after intraperitoneal injection of *P. salmonis* for the in vivo experiment in Rise et al. (2004a)). The 4K IMB microarray was also used to assess changes in gene expression in enriched macrophages from Atlantic salmon at three time points after infection by *A. salmonicida* grown in broth versus in intraperitoneal implants (Ewart et al. 2008). The GRASP 16K cDNA microarray has been used to identify Atlantic salmon gill genes responsive to the amoebic gill disease-causing parasite at four time points in the experimental challenge (0-, 44-, 114-, and 189-hour postinoculation; Morrison et al. 2006), and juvenile Atlantic salmon (whole body) gene expression correlates of saprolegniasis (Roberge et al. 2007). For details on microarray experimental design (e.g., direct comparison, reference, and cyclic) and methods (e.g., target labeling, hybridization, data extraction, and data analysis), the reader is referred to the literature (e.g., papers listed in Table 4.1) and to reviews such as Rise et al. (2007).

An in vitro study utilizing the Kuopio 1.4K microarray identified rainbow trout head kidney macrophage genes responsive to lipopolysaccharide (LPS) alone or to a mixture of LPS and cortisol (MacKenzie et al. 2006a). Gene expression of the cells was evaluated at a single time point of treatment (12 hours). While microarray

experiments arising from in vivo challenges generally identify pathogen-responsive genes at the tissue, organ, or whole organism level, in vitro studies (e.g., MacKenzie et al. 2006a) are required to capture the GERs of specific cell types (e.g., macrophages) to a pathogen or pathogen-like stimulus.

The OSU 1.6K oligo microarray and real-time quantitative RT-PCR (qPCR) were used to identify and validate rainbow trout genes responsive to killed bacteria (*Listonella anguillarum*) in adjuvant (Gerwick et al. 2007). Liver tissue gene expression was evaluated at a single time point postinjection (24 hours) and in noninjected controls. This study improved our understanding of the genes involved in innate immune responses of salmonids. It also showed that individual, sexually immature salmonids within a cultured population may have very different levels of expression of genes that play important roles in acute phase responses, inflammation, and/or immune responses (e.g., interferon inducible protein and hepcidin). This type of work could lead to the development of molecular biomarkers and tools for screening candidate broodstock to identify individuals that are capable of mounting robust innate immune responses (i.e., potentially conferring heightened resistance to pathogens and parasites).

Functional genomic studies such as these benefit aquaculture research by improving our understanding of the host genes and molecular pathways altered by contact with pathogens (e.g., bacterial, viral, fungal, and protozoal) of farmed and wild salmon. It may be useful to resequence these genes within broodstock development programs to identify single nucleotide polymorphisms (SNPs) and other sequence variants (e.g., in exons, introns, and/or regulatory regions) that may serve as molecular biomarkers for robust immune responses to pathogens and/or natural resistance to pathogens.

For experiments involving pathogen-challenged host cells or tissues, it is best to sample at various, appropriate time points in the infection process to gain a thorough understanding of the genes and molecular pathways involved in the host response. For example, salmonid immune tissue genes that respond to a pathogen such as *P. salmonis* immediately after onset of contact (e.g., 1–6 hours after initial contact between host and pathogen) may be the best candidates for development of tools and techniques for marker-assisted selection (MAS) of immune-robust families or individuals. Early-response host defense genes may encode receptors, ligands, or other components of cell signaling pathways that play key roles in determining the robustness of an innate immune response, while later-response host genes may simply be indicative of tissue damage caused by inflammation.

Chronic inflammation decreases salmonid growth rate and therefore may have a negative impact on production (Johansen et al. 2006). Various techniques, including hybridizations with the 16K GRASP microarray, were used to identify molecular pathways altered by chronic inflammation (repeated LPS injections) (Johansen et al. 2006). This study showed that rainbow trout and mammals appear to have different molecular mechanisms by which chronic immune stimulation decreases growth rate. Microarray-identified genes that were more than twofold up- or downregulated in immune-stimulated rainbow trout liver or muscle relative to saline-injected controls (e.g., calcyclin-binding protein) suggested molecular pathways (e.g., Ca^{2+}-mediated signal transduction) that may play roles in the physiological response of salmonids to chronic inflammation (Johansen et al. 2006). In the future, it is possible that molecular biomarkers of chronic stress or inflammation may be useful in developing aquaculture parameters (e.g., light regimen, temperature, and feed formulation) and standard

operating procedures that are minimally stressful and facilitate the achievement of maximal productivity.

Salmonid Growth

A recent microarray-based study used three different salmonid cDNA microarray platforms (3.5K GRASP, 16K GRASP, and 4K IMB) and qPCR to identify and validate coho salmon (*Oncorhynchus kisutch*) liver genes responsive to growth hormone (GH) transgenesis in the presence or absence of ration restriction (Rise et al. 2006). A scatterplot from one of the 3.5K GRASP chips involved in this study is shown in Figure 4.1. Meta-analysis (e.g., cross-platform comparisons of results), along with intra-platform analyses, identified suites of genes that were induced or suppressed by GH transgenesis, growth, or ration restriction. Genes that reproducibly responded to the GH transgene, such as glycerol-3-phosphate dehydrogenase and 78-kDa glucose-regulated protein (Figure 4.1), may be suitable gene expression biomarkers of impact of escaped GH transgenic individuals on wild populations. Genes found to be reproducibly dysregulated by enhanced growth (e.g., nuclear protein p8; Rise et al. 2006)

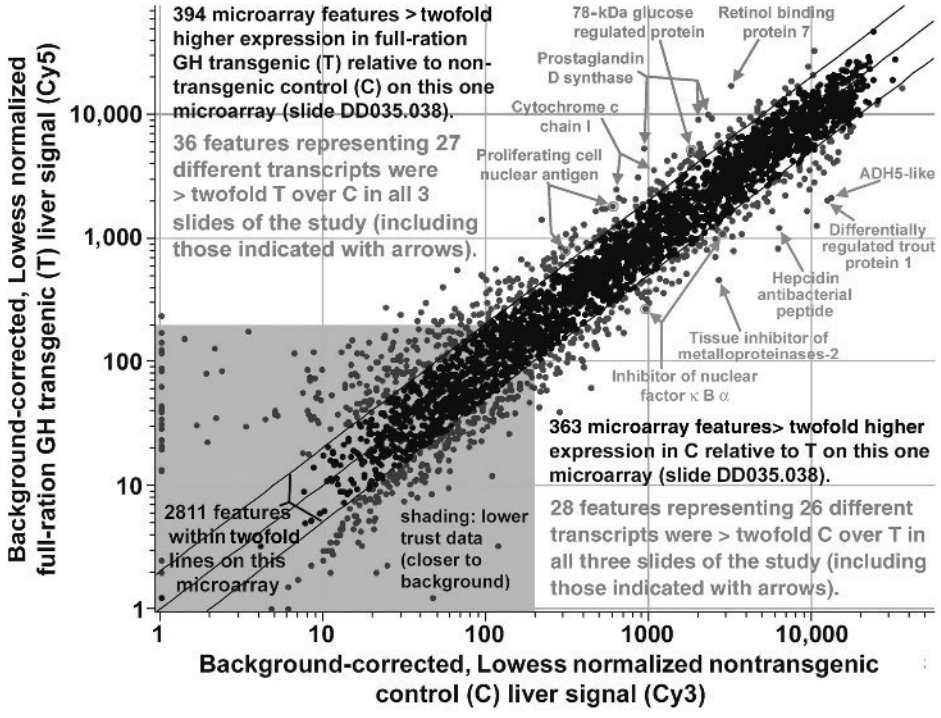

Figure 4.1. Scatterplot of background-corrected Lowess-normalized fluorescent signal data from one 3.5K GRASP microarray comparing global gene expression in full-ration growth hormone transgenic coho salmon liver with control nontransgenic liver (from Rise et al. 2006). Selected reproducibly informative microarray features (genes) are indicated with arrows and gene names (i.e., names associated with top BLASTX hits for salmonid expressed sequence tags corresponding to cDNA spots on the microarray).

may be good candidates for development of MAS tools (e.g., gene expression biomarkers of enhanced growth potential, and SNPs that may be used to identify broodstock predicted to give rise to rapidly growing progeny).

A second microarray study used a small microarray (0.5K Amago salmon cDNA) and suppression subtractive hybridization (SSH) cDNA libraries to compare liver gene expression in GH transgenic and control Amago salmon (*O. masou*) (Mori et al. 2007). This study did not look at impact of ration restriction on liver gene expression. Some similarities between the studies follow. Complement components were found to be downregulated in transgenic liver in Rise et al. (2006), and complement C3-1 was highly prevalent in Mori et al.'s (2007) reverse SSH library (enriched for genes suppressed by GH transgenesis). Lectins were found to be dysregulated by GH transgenesis in both studies (Rise et al. 2006; Mori et al. 2007). Cytochrome c (Figure 4.1) and other genes involved in mitochondrial electron transport were upregulated in GH transgenic relative to control liver in Rise et al. (2006), and cytochrome c oxidase polypeptide III was found in Mori et al.'s (2007) forward SSH library (enriched for genes induced by GH transgenesis). A notable difference between the studies is that Rise et al. (2006) found that a salmonid gene similar to delta-6 fatty acyl desaturase was upregulated in full-ration GH transgenic (T) liver relative to both ration-restricted (R) and control nontransgenic (C) tissue (expression $R < C < T$), while Mori et al. (2007) reported that a salmonid gene identified as delta-6 desaturase was downregulated in GH transgenic liver relative to control liver. Possible explanations for this apparent difference are that the two groups were looking at the expression of structurally similar but nonorthologous genes (i.e., potentially distinct regulation), that different salmonid species may have distinct responses to GH transgenesis, or that the nutritional status of the fish involved in the two studies may have differed and thereby influenced hepatic gene expression.

Salem et al. (2006) used the 16K GRASP microarray to identify rainbow trout (*O. mykiss*) muscle genes responsive to atrophy during vitellogenesis (i.e., differentially expressed in fertile and sterile female tissues). This study provided insight into the genes, and the physiological and biochemical processes, involved in vitellogenesis-induced muscle wastage in an important aquaculture species. Target genes were identified for further studies of the genetic regulation of rainbow trout muscle growth.

Salmonid Reproduction

The GRASP 3.5 K cDNA microarray was used to profile gene expression in precociously developing rainbow trout ovary (von Schalburg et al. 2005a), and this study was further described in Rise et al. (2007).

The Kuopio 1.4K microarray was used to identify rainbow trout ovary genes responsive to the Gram-negative bacterial mimic LPS at two time points postinjection (24 and 72 hours) (MacKenzie et al. 2006b). This microarray study showed that LPS downregulated several antiapoptotic genes in rainbow trout ovary (consistent with increased apoptosis seen in LPS-treated brook trout ovary), suggesting that immunogenic stimuli could alter salmonid ovary physiology and influence reproduction.

Bonnet et al. (2007) used a 9K cDNA microarray printed on nylon (Gene Expression Omnibus (GEO) platform number GPL3650) and qPCR to identify and

validate rainbow trout oocyte gene expression biomarkers of low developmental potential (assessed as percentage of normal alevins at the end of sac fry stage). Through global gene expression profiling, the authors identified genes that were differentially expressed in hormone-induced and/or photoperiod-manipulated eggs relative to naturally ovulated eggs. Functional annotations and literature pertaining to informative genes in this study revealed molecular mechanisms that could be involved in the decreased developmental potential of eggs from controlled ovulations. This type of research could lead to the development of a molecular test for low-quality oocytes. For example, since both microarray and qPCR results showed that prohibitin 2 expression was negatively correlated with normal early development (Bonnet et al. 2007), a qPCR assay for this gene could potentially be used for identifying salmonid eggs of low developmental potential.

Salmonid Nutrigenomics

The GRASP 16K platform and qPCR were used to study intestine GERs of increased or decreased phosphorus (P) levels in the diet (Kirchner et al. 2007). Four genes encoding antiviral proteins including Mx1 and viral hemorrhagic septicemia virus (VHSV)-induced protein were suppressed by P deficiency, suggesting dietary P could influence innate immune responses (Kirchner et al. 2007). Molecular biomarkers of altered P level in the diet could allow fish farmers to identify P deficiency early and to take steps to prevent the damaging effects of long-term P deficiency.

Microarray Research on Catfish

The first catfish microarray was a 660-gene channel catfish (*Ictalurus punctatus*) cDNA array printed on nylon and was used to identify channel catfish brain genes responsive to cold acclimation at four time points (0, 2, 24, and 48 hours) after a shift from 24°C to 12°C (Ju et al. 2002). Whereas experiments involving glass microarrays generally utilize fluorescence-based target labeling reagents and methods such as the Cy3- and Cy5-based gene expression kits supplied by Genisphere or Invitrogen, Ju et al. (2002) used the Digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit II (Roche). Cultured catfish in the USA must be able to tolerate wide ranges in water temperatures throughout the year. The findings of this study (e.g., transient induction of chaperone and signal transduction pathway genes) provided insight into the genes and molecular pathways altered in the catfish brain during exposure to a type of environmental stress that is relevant to the aquaculture industry (decreased ambient temperature) (Ju et al. 2002). A comprehensive understanding of the genes involved in cold acclimation may reveal suitable targets (e.g., expression biomarkers or SNPs) for MAS of cold-resistant catfish broodstock.

The construction and utilization of catfish in situ oligo microarrays are covered in detail in a later section of this chapter. Briefly, a study was run with a 19K *I. punctatus* oligo microarray platform to identify catfish spleen genes responsive to LPS at four time points postinjection (2, 4, 8, and 24 hours) (Li and Waldbieser 2006). The qPCR performed in this study confirmed the microarray platform's accuracy. This study also confirmed that many immune-relevant genes (e.g., TLR5, interferon regulatory

factor 1, and a chemokine receptor) responded in LPS-stimulated catfish spleen. Li and Waldbieser (2006) make some important points about the use of microarrays in aquaculture research, stating that such studies can (1) provide "gene expression fingerprints" (molecular biomarkers) of host immune response, pointing to "molecular pathways involved in pathogen neutralization and/or removal"; (2) "identify candidate genes controlling pathogen-specific immunity"; and (3) "identify heritable differences in gene expression levels that correlate with disease resistance/susceptibility." They go on to state that gene expression data on host responses to pathogens "would help in the formulation of a selection index to identify broodstock with superior genetic potential for resistance to disease" (Li and Waldbieser 2006).

Infectious diseases are among the most serious threats to the global aquaculture industry. Therefore, one of the main applications of fish genomic resources and techniques is to identify genes that may be suitable targets for MAS of disease-resistant broodstock. Channel catfish is the most economically important aquaculture finfish species in the USA, and the immune system and responses of this species are well characterized. While channel catfish are relatively susceptible to *E. ictaluri* infection, blue catfish (*I. furcatus*) are relatively resistant. A 28K catfish in situ oligo microarray platform was recently developed, with features representing all discrete transcripts currently known in *I. punctatus* (21K genes) and *I. furcatus* (7K genes). This is an outstanding genomics platform for comparative transcriptomic studies aimed at identifying candidate genes or heritable expression biomarkers of natural resistance to *E. ictaluri*. Two studies using the 28K catfish microarray provide a comprehensive look at the hepatic GERs of channel catfish and blue catfish to challenge with *E. ictaluri* at a single time point in the infection process (3-day postexposure) (Peatman et al. 2007, 2008). A comparison of the results of these two microarray studies identified a suite of 58 genes that responded to the pathogen in channel catfish but not in blue catfish. This differentially responsive suite included immune-relevant genes such as CC chemokine SCYA106, MHC class I alpha chain, and matrix metalloproteinase 13 (MMP13) (Peatman et al. 2008). These microarray results prompted further qPCR analyses revealing that MMP13 was 21.8-fold upregulated in channel catfish liver 24-hour 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). MMP13 may, therefore, be a suitable candidate gene for development of MAS methods (e.g., resequencing within a broodstock development program to identify SNPs, and SNP genotyping to look for correlations between MMP13 genotypes and disease resistance). These studies identified numerous genes that potentially play roles in the differential sensitivity of channel and blue catfish to *E. ictaluri*, forming "a solid foundation for future functional characterization, genetic mapping, and QTL analysis of immunity-related genes from catfish" (Peatman et al. 2008). For further information on the fabrication and application of the 28K catfish oligo microarray, see a later section of the chapter devoted to this platform.

Aquaculture-Relevant Microarray Research on Flatfish

The Bastard halibut, also called Japanese flounder (*Paralichthys olivaceus*), is an important species for the Japanese fisheries and aquaculture industries. Infectious diseases such as edwardsiellosis pose a serious threat to Japanese flounder culture

(Matsuyama et al. 2007). Several experiments have been performed using approximately 1K cDNA microarrays to identify Japanese flounder genes responsive to immunogenic stimuli including vaccines (e.g., Table 4.1). For example, genes up- or downregulated in cultured kidney cells by immunogenic stimuli such as concanavalin A, LPS, and hirame rhabdovirus (Kurobe et al. 2005) were identified. This group studied GERs at 1 and 6 hours of stimulation and confirmed their microarray results by performing conventional RT-PCR for seven genes of interest.

VHSV causes disease in various teleosts including Japanese flounder and salmonids, and VHS causes significant economic losses for salmonid aquaculture. This has spurned work on vaccines to protect Japanese flounder from the virus. Byon et al. (2006) used an approximately 1K Japanese flounder cDNA microarray platform to study the head kidney GER to recombinant glycoprotein vaccine against VHSV and compared these results to the head kidney GER to a VHSV G-protein DNA vaccine (Byon et al. 2005). The DNA vaccine was highly effective (low mortality of flounder with injection of VHSV one month after immunization), whereas the recombinant glycoprotein vaccine had low efficacy (high mortality seen in postimmunization challenges with VHSV). Comparisons of fish immune tissue GERs to effective versus ineffective vaccines may identify gene expression biomarkers of effective vaccination and shed light on the molecular mechanisms involved in protection. For example, Byon et al. found that the gene encoding interferon-inducible Mx protein was induced following immunization with the highly effective DNA vaccine (Byon et al. 2005), but not by the ineffective recombinant glycoprotein vaccine (Byon et al. 2006). A similar more recent study identified a suite of genes (e.g., ISG15, ISG56, and Mx) that were upregulated in response to an effective DNA vaccine (made from the hirame rhabdovirus G-protein gene) but not in response to an ineffective DNA vaccine (made from the hirame rhabdovirus nucleocapsid protein gene) (Yasuike et al. 2007). These results suggested that the type I interferon system must be stimulated in order for a vaccine against hirame rhabdovirus to be effective and provided molecular biomarkers of the flounder immune response to vaccines that may be useful in the development of future vaccines against viral pathogens.

Aquaculture-Relevant Microarray Research on Zebrafish

Most published studies involving zebrafish DNA microarrays employ the zebrafish as a model for studying the genetics and molecular mechanisms/biochemistry underlying developmental processes (e.g., cell fate specification and organogenesis), processes of interest to biomedical research (e.g., aging, tumor formation, and angiogenesis), and responses to environmental toxicants (toxicogenomics). These types of studies are beyond the scope of this chapter. We focus on selected studies in which zebrafish were used as physiological genomic models for studying fish responses to hypoxia, microbes, and other aquaculture-relevant stressors (Table 4.1). Advantages of using zebrafish as models for aquaculture research include the excellent genomic resources available for this species (e.g., microarrays with good coverage of the transcriptome, and a well-characterized transcriptome evidenced by the presence of more than 1.6 million zebrafish ESTs in GenBank) and their rapid development and generation times (facilitating gene function research such as morpholino-based target gene knockdown, and genetic research such as quantitative trait locus mapping). Disadvantages of using zebrafish as models include the high cost of commercially available

microarrays for this species (e.g., Affymetrix) and the small size of zebrafish tissues relative to aquaculture species, making it more difficult to perform microarray experiments on individual zebrafish for assessing biological variability.

A 4.5K zebrafish cDNA microarray and qPCR were used to identify and confirm hypoxia-responsive genes in early life stage of zebrafish (Ton et al. 2003). More recently, a 15.5K zebrafish oligo microarray platform was used to identify gill genes responsive to hypoxia (van der Meer et al. 2005). Fish and shellfish in aquaculture environments may be exposed to hypoxic conditions due to factors such as fluctuating water temperature or interrupted water flow. Therefore, it would be useful to have methods for selecting individuals that are naturally resistant to hypoxia. The genes that are identified as hypoxia-responsive may be candidates for developing MAS methods.

A 16K zebrafish oligo microarray was used to study the impact of long-term decreased ambient temperature (1 year at 18°C vs. 1 year at the control temperature of 28°C) on tail muscle gene expression (Malek et al. 2004). Three pools of individuals at 18°C ($n = 5$ each pool) were compared to three pools of individuals at 28°C ($n = 5$ each pool). This study was conducted to identify genes regulated by temperature reduction, which may be part of the molecular mechanism by which decreased temperature increases life span (Malek et al. 2004). While this is principally a biomedical application (i.e., using zebrafish as a biomedical model for research on vertebrate aging), it also benefits aquaculture research since cultured fish may also experience cold stress. Fish genes that respond to temperature reduction, and/or are differentially expressed in cold-resistant and cold-sensitive individuals or strains/families, may be good targets for developing MAS tools for selecting cold stress-resistant individuals within a breeding program.

Zebrafish 16K oligo microarrays were also used to study how the digestive tract responds transcriptionally to normal (nonpathogenic) microbes (Rawls et al. 2004). This group used microarrays to compare global gene expression in digestive tracts of germ-free (gnotobiotic) zebrafish and normal cultured zebrafish (i.e., exposed to water from a zebrafish recirculating tank system so that a normal gut microbial community could be established). They found that the normal microbiota caused changes in the expression of genes involved in innate immunity and nutrient metabolism. Importantly, the germ-free zebrafish experienced upregulation of genes associated with impaired nutrient utilization and fasting relative to the zebrafish exposed to normal (commensal and/or symbiotic) bacteria. The normal gut microbial communities of zebrafish (Rawls et al. 2004) influence phenotypic traits of interest to the aquaculture community (e.g., nutrient utilization, growth, and innate immunity). Therefore, it is not simply the genetics and genomics of the fish (host) that we should be studying to improve production, but also the genomics of associated microbes and host–microbe interactions. These ideas are further developed in a later section of the chapter dedicated to the development and use of genomic tools for studies of host–symbiont interactions, and the potential impact that this type of research may have on the future of aquaculture research.

The Use of DNA Microarrays to Investigate Aquatic Animal Pathogens

Genomics and proteomics technologies are increasingly being used to investigate the mechanisms of pathogenicity of aquatic animal pathogens. One of the tools showing

great promise is the DNA microarray, which can be used as a tool to diagnose, characterize, and determine species of bacteria, or other aquatic animal pathogens, including the determination of the degree of genetic variation among strains of aquatic animal pathogens (Gonzalez et al. 2004; Ong et al. 2004; Taboada et al. 2004; Nash et al. 2006).

To date, few DNA microarrays for aquatic animal pathogens have been constructed. Microarrays containing red sea bream iridovirus (Lua et al. 2005; Thi et al. 2007), Singapore grouper iridovirus (Chen et al. 2006), and shrimp white spot syndrome virus (Marks et al. 2005; Lan et al. 2006) open reading frame (ORF) DNA sequences have been used to study viral gene expression changes occurring during infections. A partial and full-genome array was constructed with amplicons of genes of *A. salmonicida* subsp. *salmonicida* (Nash et al. 2006; see later section of this chapter for details on the fabrication of this microarray platform), and these have been used for the microarray-based comparative genomic hybridization (M-CGH) as described above.

Gonzalez et al. (2004) have used oligo-based DNA microarrays to detect aquatic animal and other pathogens, including *Vibrio vulnificus*, *L. anguillarum*, *Photobacterium damselae* subsp. *damselae*, *A. salmonicida* subsp. *salmonicida*, and *V. parahaemolyticus*.

In addition, DNA microarrays can be used to investigate gene expression of pathogens when grown under specific conditions, or at specific stages of infection. DNA microarrays have been used to examine transcription profiles of some human pathogens such as *Mycobacterium tuberculosis* (Talaat et al. 2000; Manganelli et al. 2001), *Salmonella enterica* (Eriksson et al. 2003), and *Campylobacter jejuni* (Carrillo et al. 2004). However, to our knowledge, Brown et al. (personal communication) is the only group thus far to use microarrays based on the pathogen DNA or RNA to investigate transcriptional profiles of bacterial pathogens in aquatic animals. Several studies have used cDNA microarrays for investigation of fish gene expression (e.g., Rise et al. 2004a; Byon et al. 2005; Ewart et al. 2005); however, there is little information on pathogen gene expression using microarray technologies.

Examples of Novel Microarray Platforms for Aquaculture-Related Research

Microarrays are, in principle, simply high-density dot blots. Their use for the analysis of gene expression often provides coverage of a good proportion of the entire genome. The basic question for the design, fabrication, and application of microarrays in a given species most often depends on what genome resources are available for the species of interest. This issue is most relevant with respect to aquaculture species as genome resources for many aquaculture species are still limited. For instance, for the best design of microarrays, whole genome sequences are needed to include all potential genes and to avoid the inclusion of repetitive sequences among features (target sequences). Obviously, with the exception of just a few aquaculture species, this requirement will not be met for most of the several hundred species used in aquaculture. In order to provide a good coverage of the entire genome expression, or expression involving specific pathways, a large number of genes must be known for the species of interest. Such information is generally provided by development of EST resources. With the gene sequence information in hand, the researchers are then faced with considerations of the design, fabrication, and application of microarrays.

Microarray Example 1: Catfish In Situ Oligo (Contributed by Zhanjiang Liu)

The Intention for Developing the Catfish Microarray Platform

This section provides some of the elements we considered for the design, fabrication, and application of the catfish microarrays. The first question facing us was the purpose of the catfish microarray. In spite of their application for many different purposes, the relative high costs of microarrays have been the limitation for their applications. Keeping the cost issue in mind, we decided to develop a microarray platform that included the largest number of genes covering the largest possible transcriptome of channel catfish (*I. punctatus*). Such a platform can be used as a screening tool for various differentially expressed genes under various "treatments" (here the word "treatment" is used to include all comparisons between different genotypes, between infected samples and healthy samples). Thus, our first task was to develop an EST resource for catfish representing the largest possible number of genes in absence of the whole genome sequences in catfish.

We are also interested in developing a large EST resource from blue catfish (*I. furcatus*) as most of our genome research effort has been on using the channel catfish × blue catfish interspecific hybrid system. Blue catfish is a closely related species that can produce fertile F_1 hybrid with channel catfish. It possesses several superior performance traits to those of channel catfish, including greater disease resistance to enteric septicemia of catfish (ESC, the most severe bacterial disease in catfish), a more uniform body shape allowing a greater processing yield, and greater seinability. Inclusion of features representing blue catfish will allow studies of genome expression of both species under various experimental conditions.

The Development of the Catfish EST Resources

Several considerations were made for the development of the catfish (both channel catfish and blue catfish) EST resources. First and foremost, the gene discovery rate must be high in order to identify the largest number of possible genes with limited sequencing. To satisfy this demand, we used a combined strategy of both normalization and subtraction, coupled with negative-hybridization selection for novel clones (Li et al. 2007). Second, the EST resources should provide us with the ability to detect SNPs. To satisfy this requirement, we used multiple individuals representing various genetic backgrounds to include as much SNP information as possible in the cDNA libraries (Ju et al. 2000; Karsi et al. 2002; Li et al. 2007). Third, we need to be able to identify genes induced after infection as disease resistance and innate immunity is a high priority of our research. We included both healthy tissues and infected tissues for the construction of our cDNA libraries used for EST sequencing (Cao et al. 2001; Kocabas et al. 2002).

To date, we have sequenced more than 40,000 channel catfish ESTs representing more than 21,000 unique sequences, and more than 10,000 blue catfish ESTs representing more than 7,000 unique gene sequences. Working with the Joint Genome Institute (JGI) of the Department of Energy (DOE), we are producing 400,000 more channel catfish ESTs, and 200,000 blue catfish ESTs (unpublished information).

The Features (Gene Probes) of the Catfish Microarray Platform

Here, we describe a 28K catfish microarray. We must first give credit to Robert Li and Geoff Waldbieser for their role in the development of the 19K version of the catfish microarray (Li and Waldbieser 2006) mentioned earlier in this chapter. Our goal with the newer, 28K microarray platform was to include all known genes from catfish with the largest possible coverage of the transcriptome of both channel catfish and blue catfish. Cluster analysis was used to determine the unique gene sequences. All existing catfish ESTs allowed identification of 21,359 unique gene sequences from channel catfish and 7,159 unique ESTs from blue catfish (Peatman et al. 2007, 2008). All these were included on the catfish microarray.

To obtain a unique set of blue catfish ESTs, all sequences available in the NCBI GenBank for the species as of March 2005 were downloaded in FASTA format, added into the ContigExpress program of the Vector NTI software suite (Invitrogen, Carlsbad, CA) and assembled. Singletons (nonclustering sequences) and representative clones from contigs were selected and reassembled in ContigExpress to ensure a unique gene set as described previously by Peatman et al. (2004). A total of 28,518 sequences were used to construct the new catfish microarray. The added channel catfish and blue catfish sequences were compared by BLASTX against the nonredundant (*nr*) protein database at NCBI, with a cutoff E value = 0.00001 for annotation. A record of all sequences contained on the 28K catfish microarray, their putative identities, expression values on each slide, and other experimental data have been deposited in the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) accessible through the GEO series accession number GSE6105.

BLAST searches are highly recommended in addition to cluster analysis. That is because in many cases, two or more short ESTs can exist in the dbEST and because they do not overlap, cluster analysis alone would characterize them as two or more unique gene sequences, but they truly represent a single transcript. BLAST analysis would allow the flags to be raised; thereby, less redundancy is involved. However, as microarrays are most often used as a screening tool, the presence of more features on an array should not prevent further characterization, except that the data may be initially inflated until additional characterization and resolution.

The Design and Fabrication of the Catfish Microarray Platform

Several major platforms were considered for the design and fabrication of the catfish microarray including the in situ oligo array, spotted oligo array, and the spotted cDNA microarray. Spotted arrays are constructed by spotting long oligos or cDNAs using a printing robot, whereas in situ arrays are constructed by synthesizing short oligos directly onto the slide by photolithography. Our decision of using an in situ oligo array platform was based on the following considerations: (1) the purpose of the microarray and the reproducibility of the array, (2) the cost of the platform and the available financial resources, (3) the number of intended users, and so on. As discussed above, we intend to use it as a screening tool for differentially expressed genes in both channel catfish and blue catfish. In terms of precision and reproducibility, the in situ arrays perhaps provide better results, although opposite opinions do exist. In general, however, spotted arrays tend to bring in more variations among repeated spots on the array. In situ arrays are more expensive on the per array basis, but the one-time

investment of a spotted array involving a large number of genes may be beyond the capacity of a small laboratory without a special grant, although the cost on the per-array basis is lower. Oligo- or cDNA-based arrays each have pros and cons, but oligo-based arrays have no need of accurate clone tracking and do not require the availability of the cDNA clones for amplification of the inserts. Spotted arrays would provide the possibility of producing a large number of arrays with relatively low cost per array slide, and this would be particularly attractive to situations involving many users.

In the absence of the genome sequence, one prominent issue is what sequences should be included in the features. Obviously, cDNA arrays would allow long sequences to be included, while oligo arrays typically involve 24- to 70-mer oligos. Even for the oligo arrays, the portion of the gene to which the oligos should be designed is important. Some researchers favor the use of coding regions, while others favor the use of the 3′-untranslated regions (3′-UTR). The key issue here is to provide as much gene-specific hybridization as possible. Use of the coding region sequences obviously may result in cross-hybridization among genes in multigene families, or among genes sharing functional domains. In contrast, use of 3′-UTR region may, while providing greater discrimination among individual genes within multigene families, also increase the chances of inclusion of repetitive elements in the features, especially in the absence of a draft genome sequence. In our case, we believe that complications caused by cross-hybridization of genes within multigene families can be dealt with after the initial screening stage using microarrays.

Our catfish microarray feature sequences were selected by Nimblegen, which designs probes indiscriminately across available sequences to provide the highest "uniqueness" with existing information. Obviously, the more sequence information is available from a species, the better the "uniqueness." Nimblegen Systems produced the physical microarrays utilizing an in situ maskless array synthesis technology to synthesize 24-nucleotide (24-mer) oligos on the surface of the microarray slides (Singh-Gasson et al. 1999; Nuwaysir et al. 2002). At least twelve 24-mer oligos were designed for each EST represented on the microarray. Half of these were perfect-match oligos selected along the length of the sequence, while the other half were duplicates of the first but with two mismatched bases at the number 6 and number 12 positions (Peatman et al. 2007, 2008).

To date, we have used the catfish microarrays to study differentially expressed genes between channel catfish and blue catfish, both before and after bacterial infection (Peatman et al. 2007, 2008). Our results suggested high levels of reproducibility and usefulness of the arrays, especially for genes that are highly induced (Table 4.2). However, the largest limitation continues to be the high cost involved in the use of in situ oligo-based arrays. Recent technological advances and industry competition have allowed significant reduction of the cost of microarrays, but to many underfunded aquaculture researchers, they are still too expensive to be frequently used.

In summary, in considering the design of different platforms of arrays for a given aquaculture species, key issues are as follows: (1) is the genome sequenced and annotated?; (2) if the genome sequence is not available, how many ESTs are available and how many genes do they represent? Answers to these two basic questions would allow the determination of the optimal number of features to be included on the microarray. Obviously, no matter what genes are placed on the array, the greater the number of genes, the more powerful the microarrays are for the purpose of gene expression studies. However, specific arrays can be designed for specific purposes. For instance,

Table 4.2. Catfish genes upregulated fivefold or greater in the liver following infection of *E. ictaluri*.

Accession	Putative identity	Function	Fold change	*q* value
CF970955	Intelectin	Pathogen recognition; iron metabolism	85.4	1.25
CK408483	Haptoglobin precursor	Binds hemoglobin; APP	34.3	0.00
BM438750	Microfibrillar-associated protein 4	Unknown; lectin similar to ficolin and tachylectin	32.9	1.25
TC6845	Intelectin	Same as above—putative paralogues	28.0	2.36
BM438689	Microfibrillar-associated protein 4	Same as above—putative paralogues	25.6	0.00
TC8425	Warm temperature acclimation-related 65-kDa protein-like protein	Similar to hemopexin—sequesters heme	23.4	0.00
TC7475	CC chemokine SCYA113	Unknown; putative catfish orthologue of human CCL19/ MIP-3-beta	21.5	3.27
CK406396	Neurotoxin/C59/Ly-6-like protein	Unknown; possible phospholipase inhibitor or complement membrane attack complex inhibitor	21.3	1.25
CV994031	Catechol-*O*-methyltransferase domain containing 1	Unknown; putative *O*-methyltransferase	14.8	0.00
TC9205	Hypothetical protein XP_683888	Unknown	14.4	1.25
TC8426	Hemopexin precursor	Sequesters heme to liver	13.6	2.36
CV996638	Apolipoprotein Apoa4 protein	Lipid binding and transport	13.0	2.36
CV993724	Toll-like receptor 5	Pathogen recognition receptor—flagellin	11.8	0.00
CV987901	Complement C3-H1	Complement pathway; inflammation	10.0	1.71
EE993362	Complement protein component C7-1	Membrane attack complex component	9.7	0.00
TC9637	Fibrinogen alpha chain	Coagulation factor; APP	9.6	0.00
TC9194	Complement regulatory plasma protein	Factor H; complement inhibition	8.9	3.27
CV992853	Ceruloplasmin	Iron transport; APP	8.5	0.00
TC9833	Microfibrillar-associated protein 4	Same as above—putative paralogues	8.4	1.25

(*continued*)

Table 4.2. (*Continued*)

Accession	Putative identity	Function	Fold change	*q* value
TC8765	Transferrin	Transports iron; APP	7.7	0.00
TC8306	Fibrinogen gamma polypeptide	Coagulation factor; APP	6.1	0.00
CV989503	CXCL14	Chemokine—stimulates monocytes, NK cells	5.7	0.00
CV997126	Complement C3	Complement pathway; inflammation	5.6	0.00
TC7892	Ceruloplasmin	Same as above—putative paralogues	5.4	0.00
CV992447	Complement component C8 beta	Membrane attack complex component	5.4	3.27
TC7741	Complement factor B/C2-A3	Complement pathway	5.4	3.74
BM494620	Serum/glucocorticoid-regulated kinase	Cellular stress response	5.3	1.25
CV995884	Solute carrier family 31 (copper transporters), member 1	Copper ion transport	5.3	0.00
TC8490	Fibrinogen, B beta polypeptide	Coagulation factor; APP	5.2	1.71
EE993545	Erythroblast membrane-associated protein	Cell adhesion or receptor molecule of erythroid cells; Ig superfamily member	5.0	3.74

Accession refers to the GenBank accession number or TIGR consensus number. *q* value is the false-discovery rate for the particular gene.

pathway arrays can be designed to include only those genes important to the specific pathway. Since we are more interested in global gene expression than in specific pathways at present, we will continue to focus on the fabrication of a catfish microarray providing the maximal power for this application. Considering the availability of additional gene or EST information, microarray platforms should be updated whenever the number of unique genes is significantly increased in the species. In our case, we need to design and manufacture the next generation of catfish microarrays after the completion of the JGI catfish EST sequencing project.

Microarray Example 2: Halibut Oligo (Contributed by Susan Douglas)

Atlantic halibut is highly prized for its firm, white, good-tasting meat and is currently produced commercially in Norway, Iceland, Scotland, and Canada. Halibut farming is a relatively new venture in Atlantic Canada, with the first commercial production starting in 1998. Halibut farming is a complex process; developing larvae are incubated

in a series of land-based nursery tanks until they are large enough to be transferred to larger grow-out facilities. After approximately 3 years they are of marketable size (about 3–5 kg). Although progress has been good, significant gains in production can be made by improving our knowledge of the basic biology of this animal and by selective breeding of individuals with desirable traits.

Atlantic halibut undergoes a complete body transformation as it develops from the newly hatched larva through to the fully metamorphosed, juvenile stage (Lewis and Lall 2006). This involves eye migration (Saele et al. 2003), body flattening, skin pigmentation (Naess and Lie 1998), and organ rearrangements (Saele et al. 2004). Abnormalities in this developmental process and a number of other production-related problems involving reproduction, nutrition (Naess and Lie 1998; Hamre et al. 2005), and immunity require a better understanding at the molecular level. This has been the focus of the Genome Canada-funded Pleurogene project (www.pleurogene.ca), which aims to enhance flatfish aquaculture using a genomics approach.

As a starting point for this project, normalized cDNA libraries were constructed from five different larval stages from mouth opening to postmetamorphosis and from eight different tissues from adult fish (testis, ovary, liver, head kidney, spleen, skin, gill, and intestine). Approximately 1,000 randomly picked clones from each library were sequenced from the 5′ end, annotated, and a publicly available searchable database was developed to access this information (Douglas et al. 2007). After contigging, a unigene set was selected for design of oligo probes for a high-density microarray.

The unigene set consisted of halibut contigs (2,548) and singletons (5,805) derived from the Pleurogene project as well as additional single genes of interest that were cloned in our laboratory. Approximately 65% of these genes are annotated. Additionally, all Atlantic halibut sequence data from other research groups that were present in GenBank were assembled into contigs and unique genes (904) that were different from those that we had derived were used (Table 4.3). Most genes are represented by one oligo only, but several had oligos designed to different portions of the gene. All oligos were printed in side-by-side quadruplicates in 48 subgrids each containing 26 rows and 32 columns to give 39,936-feature microarrays. Controls included an oligo based on chlorophyll synthetase G4 from *Arabidopsis thaliana* (GenBank accession number U19382) and spots containing only buffer.

Oligo DNA probes were designed using ArrayDesigner software (Premierbiosoft, Palo Alto, CA) and were selected to have minimal secondary structure, a GC ratio between 40 and 60%, a melt temperature of 75 ± 5°C and to be 45- to 55-mer in length.

Table 4.3. Features present on Atlantic halibut microarray.

Category	Unique features	Total features
Pleurogene contigs	2,548[1]	10,572
Pleurogene singletons	5,805[1]	24,168
GenBank gi	904[1]	3,680
Single genes	20	80
Arabidopsis	1	92
Blank	1	1,344
Total	9,279	39,936

[1]Genes have more than one oligo designed on them.

Uniqueness of the selected probes was ensured by BLAST comparison against all of the halibut ESTs and the zebrafish genome. Probes were synthesized by the phosphoramidite method at a 40 nmole scale on a PolyPlex 2 (Atlantic Microarray Facility, Moncton, NB) and analyzed by ESI-MS using an Agilent VL1100MSD (Agilent, Mississauga, ON). DNA of low quality was resynthesized. Solutions (20 μM) in a sodium phosphate buffer (Schott-Nexterion Spot, Mainz, Germany) were prepared in 384-well plates (Genetix X7020, Boston, MA) and spotted on epoxide microarray slides (Schott-Nexterion) using an OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI) equipped with SMT-S50 silicon print pins (Parallel Synthesis Technology, Santa Clara, CA). Any defects that may have occurred in spotting were analyzed by SpotQC (IDT Inc., Coralville, IA).

The microarray was first tested using a self–self-hybridization with a reference RNA isolated from all five developmental stages. RNA (7.5 μg) was converted to cDNA and labeled using Alexa dyes and the Invitrogen Direct labeling kit (Invitrogen, Burlington, ON). Excluding flagged features (those with intensities under 500 pixels, control features), the majority of the features had a log ratio close to unity (Figure 4.2). Of the features that deviated from this value, none were represented by all four of the spotted quadruplicates and were considered anomalous signals.

Preliminary experiments have been performed on halibut larvae from different developmental stages. In this study, three pools of larvae were sampled from each of the five developmental stages: hatching (1 dph), mouth-opening (21 dph), midway to metamorphosis (64 dph), premetamorphosis (91 dph), and postmetamorphosis (104 dph). Two biological replicates each consisting of RNA from a given developmental stage compared to the reference RNA were analyzed. In these experiments, between 8,525 and 13,233 features (median 11,770) gave signals in both channels that were greater than 500. The Atlantic halibut microarray yields reproducible and reliable data and will be of value in identifying key genes involved in the process of metamorphosis (Douglas et al. 2008). This will lay the groundwork for understanding developmental abnormalities that sometimes occur in intensive rearing situations.

Nutrigenomic studies in fish are providing interesting insights in response to dietary changes (Jordal et al. 2005). Two halibut nutrigenomic studies are under way: one focuses on the changes in gene expression associated with the use of microparticulate diets at various stages of development, and the other aims to identify gene expression changes induced by the replacement of fish meal by soybean meal.

The use of live feed in the rearing of larval marine fish is very expensive and labor-intensive (Cahu et al. 2001). Replacing live feed with inert, microparticulate diets could result in considerable economic gains if similar growth can be obtained. We have sampled larval halibut at various times after introduction of microparticulate diet and will use the microarray to elucidate whether expression of genes involved in key metabolic pathways is adversely affected.

With the worldwide supply of fish meal (a required component of aquaculture feeds) rapidly declining, attempts are being made to utilize alternative protein such as soybean meal that will result in similar growth. In salmonids, inclusion of soybean meal results in inflammation of the distal intestine (Bakke-McKellep et al. 2007); however, it is not known if similar adverse effects occur in Atlantic halibut. We have sampled juvenile halibut at three times after introduction of diet containing soybean meal and will use the microarray to evaluate changes in gene expression in the gastrointestinal tract that may be indicative of inflammatory responses or other detrimental processes.

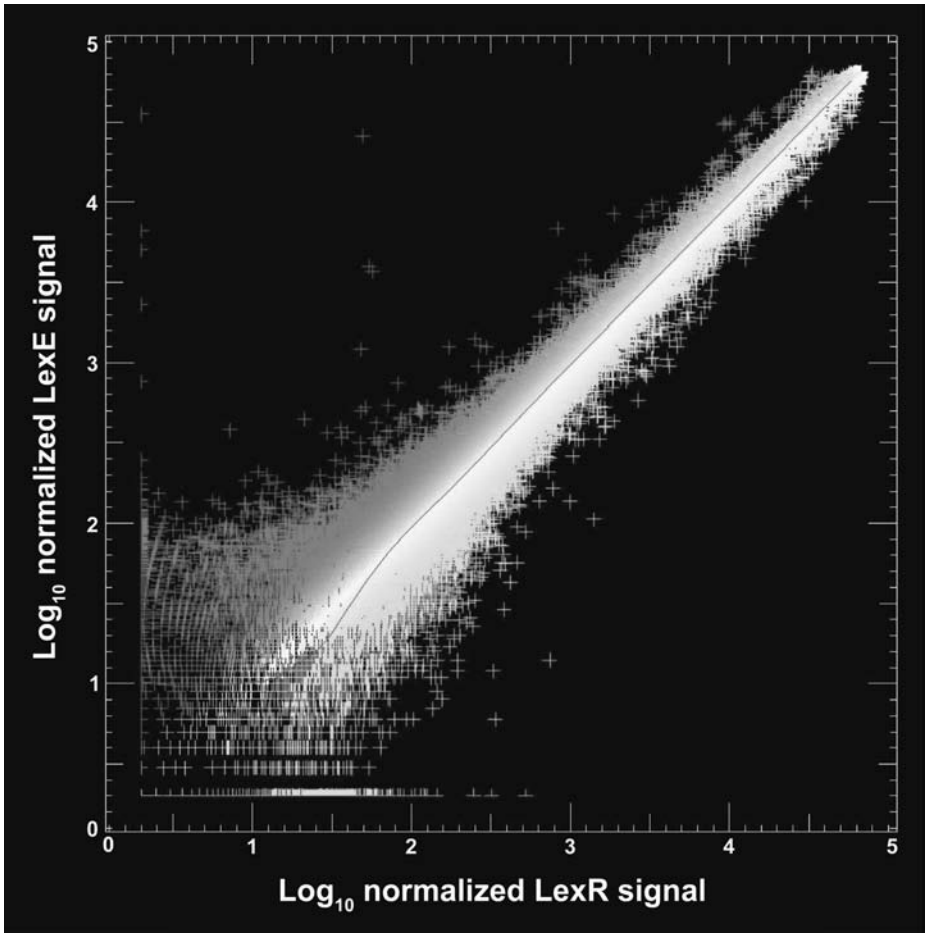

Figure 4.2. Scatterplot of Lowess-normalized data from self–self-hybridization using Atlantic halibut reference RNA.

Microarray Example 3: Aeromonas salmonicida cDNA (Contributed by Laura Brown and John Nash)

The Use of DNA Microarrays to Investigate the Pathogenicity and Genetic Diversity of the Fish Pathogen A. salmonicida

A. salmonicida, the causative agent of furunculosis, is a nonmotile, Gram-negative bacterium, and one of the most studied bacterial pathogens of fish. Furunculosis is an important disease in wild and cultured stocks of fish with the potential for severe negative economic impact. The Canadian Aquaculture Industry Alliance estimated the total direct and indirect costs of infectious diseases within the Canadian aquaculture industry at more than $400 million annually, with furunculosis contributing approximately $4 million annually to these losses (http://www.aquaculture.ca/). *A. salmonicida* is not limited to salmonids and many species of fish are affected. Typing

various isolates of *A. salmonicida* has proved to be problematic, and typing schemes based on biochemical differences or on single-gene assays are not satisfactory. We demonstrated the efficacy of microarray based comparative genomic hybridization (M-CGH) in studying genetic relationships between *Aeromonas* species, subspecies, and strains based on gene conservation profiles, and we examined the genomic diversity of strains and isolates from selected geographic areas and host species to explore correlations between geographic or host origin and conservation or diversity of genes (see Figure 4.3; Nash et al. 2006). This approach has been shown to be successful with *C. jejuni* (Taboada et al. 2004) and among virulent and avirulent *Burkholderia* species (Ong et al. 2004) as well. Bacterial DNA microarrays can also be used to examine transcript profiles, to investigate gene expression of pathogens or bacterial species under select conditions. In our laboratories, we use the *A. salmonicida* DNA microarray to investigate the mechanisms of pathogenicity by looking at differences in gene expression at selected stages of infection, and from selected host tissues, and from bacterial cells grown under specific conditions such as iron-restricted media.

The genome of *A. salmonicida* subsp. *salmonicida* strain A449, a wild-type virulent clinical isolate, has been sequenced and is deposited in GenBank (accession numbers NC009348-009350 and NC004923-004925). We constructed two DNA microarrays for our investigations of *A. salmonicida.* The first was a partial genome array. This was constructed before the sequence of *A. salmonicida* was completely finished, and information was obtained from a draft of the genome sequence. In constructing the first DNA microarray, we were constrained by the fact that the genome sequence at the time was neither complete nor fully annotated. Therefore, we selected known and putative *A. salmonicida* virulence genes, including the S-layer proteins, Types II and III secretion system proteins, siderophores, iron-restriction proteins, and surface proteins. The second DNA microarray was constructed once the entire genome sequence was known, and it contains all genes from the wild-type strain used for the sequencing project.

To construct the DNA microarrays, PCR primers were designed successfully for each of the 2,024 ORFs described above using the Primer3 program (Rozen and Skaletsky 2000) controlled by an automated script as described previously (Taboada et al. 2004). Primer-selection parameters were standardized and included a similar predicted melting temperature ($62 \pm 3^{\circ}C$), uniform length (21 nt), and a minimum amplicon size of 160 bp. Generation of PCR amplicons and fabrication of DNA microarrays were as described (Taboada et al. 2004). For the full genome array, the amplicons were all constrained to be minimum 160 bp, maximum 1,600 bp in length, and if an ORF was longer than 1,600 bp, the amplicon was split into nonoverlapping sections.

Control spots on the array consisted of empty spots, whole genome DNA from *A. salmonicida*, human DNA, and salmonid DNA. All genes were spotted in duplicate on the arrays.

Microarray Example 4: Host–Symbiont (Contributed by Margaret McFall-Ngai)

Beneficial Bacteria in Health

In this portion of the chapter, we consider the application of microarray studies to beneficial animal–bacterial interactions. The recognition that most, if not all, animals

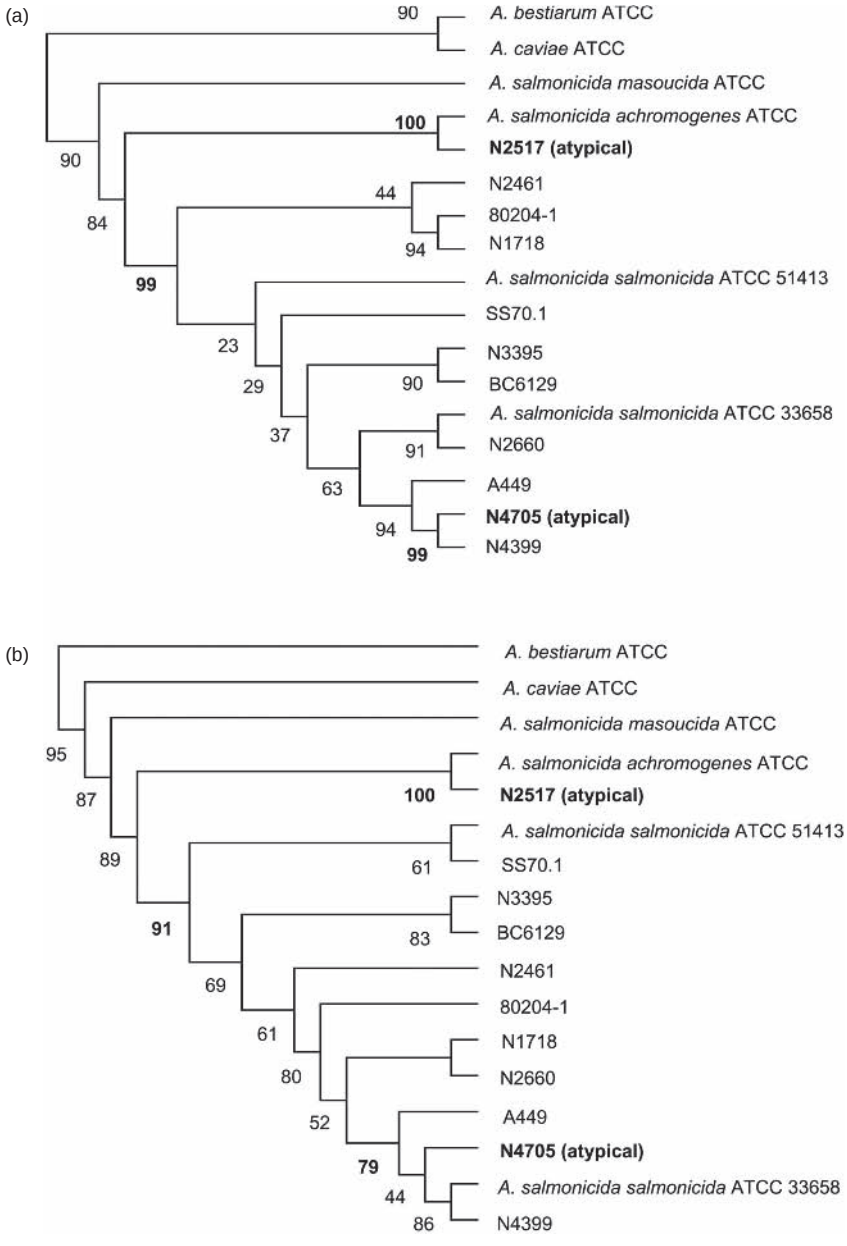

Figure 4.3. Hierarchical clustering of *Aeromonas* strains based on microarray-based comparative genomic hybridization (M-CGH) data for all genes on the microarray (from Nash et al. 2006). Tree data was calculated using the MEV software from TIGR with Euclidean distance and average linkage clustering. Bootstrapping with 1,000 replicates was used to generate support trees, and the bootstrap values are shown for each branch. Isolates in bold are atypical isolates that cluster with other known subspecies. The bootstrap values that lead to their cluster assignment are also in bold. All ATCC-type strains are denoted "ATCC," and unless otherwise noted, all other isolates are *A. salmonicida* subsp. *salmonicida.* (a) Sample clustering based on all genes on the AsalChip1 microarray. (b) Sample clustering based on genes not assigned to the plasmid or transposon functional categories (i.e., "chromosomal" genes).

likely require associations with coevolved microbial partners for health is relatively new, so a limited set of studies is available for consideration. Microarrays have been used to characterize this phenomenon in a few animals, but such approaches have not yet been used widely with aquaculture species. Thus, the following discussion can be considered horizon analysis in which we present the current state of this field and how this conceptual construct might be applied to aquaculture. We will conclude with a brief description of how microarray analysis is informing our understanding of one specific model of animal–bacterial interactions, the squid-vibrio model.

Our First Glimpses of the Frontier

Recent advances in molecular biology, particularly high-throughput sequencing, metagenomics, and microarray analyses, have been critical in enabling the study of the interactions that animals have with coevolved bacterial partners. An understanding of such relationships has been impeded by the nature of the associations; specifically, the majority of the microbes are not yet culturable in the laboratory and they often occur in highly diverse consortia. The application of the new culture-independent approaches has allowed biologists to begin to define the microbial phylotypes that live with a given animal species and to characterize their activity.

Whereas these avenues of investigation are now technically possible, the first such studies of animal systems, particularly those of vertebrates, have revealed a complexity previously not appreciated. The best-studied subjects at present are mammals (e.g., Ley et al. 2008). Analysis of the mammalian microbiota has demonstrated that bacteria occur in predictable communities associated with microenvironments in eight of the ten vertebrate organ systems (Dethlefsen et al. 2006). Characterization of the consortia in the oral cavity alone, just one region of the digestive system, has shown distinct microbial communities in seven independent niches (e.g., subgingival space, tongue, and cheek) with a total of more than 700 phylotypes of bacteria overall (Paster et al. 2006). Thus, these communities can be highly diverse and have well-defined habitat loyalty. In addition, it is likely that they usually undergo a developmental succession. Specifically, these communities are acquired each generation through "horizontal transmission," that is, the embryonic development of vertebrates occurs in the absence of their microbial partners, but not without their indirect influence; during embryogenesis, receptors are expressed that allow the animal to interact with specific microbes upon birth or hatching. A recent study of humans has demonstrated that the first years of life involve dramatic shifts in the microbial community composition of the gut tract, a maturation process that correlates with developmental progression of host tissues (Palmer et al. 2007).

These data demonstrate the complexity of animal–bacterial interactions and highlight some specific challenges. How does one go about demonstrating whether a particular community, or individual member of a community, is or is not coevolved with the host? How does the form and function of the coevolved set of microbes interface with the "tourist" population, or those microbes just passing through (e.g., coming in on the food)? These areas are currently under active investigation and, in the case of animals with complex consortia, these questions are proving to be more challenging that expected. Despite these complications, the early studies of these

interactions in vertebrates have provided such compelling evidence of the importance of animal–bacterial interactions that the National Institutes of Health has recently identified the study of the human microbiome as one of two components of its new "roadmap," i.e., targeted areas of research effort (http://nihroadmap.nih.gov/roadmap15update.asp). This decision was made in response to the recognition that biomedicine has been focusing on the mechanisms of microbial pathogenesis in the absence of an understanding of the "normal" condition, i.e., the mechanisms underlying the dynamics of the interactions of the normal microbiota with the host. It is likely that an understanding of such concepts in aquaculture will be critical to defining the healthy condition of cultured species, as well as determining what occurs when pathogens compromise health, i.e., when they encroach upon the preexisting "conversations" that a host animal has with its beneficial microbial partners.

The Nature of the Partnerships

To gain a relatively sophisticated understanding of how animals interact with microbes will require reliance on a wide variety of animal species. Similar to developmental biology, model systems, i.e., those with particular features that render them experimentally tractable, can be characterized in depth, and the knowledge obtained can be applied to other species. Comparison of a wide array of models can identify elements conserved over evolutionary time, i.e., the core set of ancient, shared responses, as well as provide insight into how diversity among systems, such as different symbiotic associations, is achieved. Both vertebrate and invertebrate models have been exploited for such analyses, and should provide heuristic value to studies of vertebrate and invertebrate aquaculture species.

Vertebrates

To approach the study of the molecular dialogue between a host and its microbial partners, it is critical to have some understanding of the "players," and the patterns of their occurrence. Once the partners are known, one can determine whether microarray analysis would be feasible and informative in studying genomic responses of one or both partners. Evidence to date suggests that it is a derived character of gnathostome vertebrates to harbor complex consortia, at least in the gut (McFall-Ngai 2005). Whereas microbiologists now recognize several dozen divisions of bacteria, the majority of the diversity of the microbes that appear to coevolve with vertebrates is found in radiations in only a small subset of the lineages; the vertebrates studied to date have consortia dominated by phylotypes within four to five bacterial divisions (Eckburg et al. 2005; Dethlefsen et al. 2007). Interestingly, most bacterial pathogens also occur within these divisions, and within a given host, pathogens are often congeners of the constituents of the normal microbiota (Salyers and Whitt 2001). These findings further support the idea that pathogens are interlopers in the preexisting conversation that a host has with its evolved, beneficial partners.

Whether the bacterial partner is beneficial or pathogenic, the complex nature of the vertebrate microbiota renders their study by microarray analysis unfeasible. However, the genomic responses of vertebrate hosts, that is, the principal eukaryotic partner, to interactions with their microbiota have been under intense investigation in the past

few years. Most notably, microarray analyses on gut tissues of zebrafish and mouse in response to the germ-free state, to interactions with specific components of the microbiota, and to interactions with the entire consortium have provided great insight into the impact of these interactions on many aspects of biology, particularly host developmental biology and nutrition (for review, see Cheesman and Guillemin 2007). Zebrafish and mouse are ideal vertebrate subjects, as they offer the opportunity for genetic manipulation of the host animal. In addition, the influence of the microbiota has been studied at other levels in these systems so that the results of genomic studies might be correlated with particular phenotypes. For example, interactions with the microbiota shortly after birth drive the development of the vasculature of the vertebrate intestine (Stappenbeck et al. 2002), and these changes can be correlated with specific symbiont-induced changes in relevant genes (Rawls et al. 2004). In the larger picture, studies of the zebrafish and mouse models have shown that the microbiota influence the regulation of a few hundred host genes. Interestingly, these two distantly related vertebrate species share dysregulation of nearly 60 genes in response to interactions with their microbiota (Rawls et al. 2004). This group of transcripts likely represents the set of genes conserved in the vertebrates for interactions of bacteria with the apical surfaces of epithelia of the vertebrate gut.

Invertebrates

Ninety-six percent of the diversity among the animals occurs in the invertebrates, and they have a wide variety of symbiotic associations. They differ from vertebrates in often harboring beneficial intracellular bacterial symbionts (Douglas and Raven 2003); while a number of intracellular pathogens associate with vertebrates, no beneficial intracellular partners have yet been identified in vertebrate tissues. In addition, many invertebrate species have monospecific bacterial associations, i.e., intra- or extracellular symbioses with populations of a single bacterial phylotype. A few such binary extracellular partnerships occur in fishes (Haygood 1993), but no such relationships exist in the tetrapods to the authors' knowledge. Finally, even if consortia are present, they may be limited to a few phylotypes. Thus, whereas a vertebrate may have several hundred phylotypes, an invertebrate may have only a dozen or so in a region of comparable dimensions (Broderick et al. 2004; Cox and Gilmore 2007). In addition, experimental manipulation of certain insect species has demonstrated that the coevolved set of microbes within this population may be limited to two or three phylotypes (Broderick et al. 2004). Similarly, a recent study of the microbiota associating with two hydra species has demonstrated a highly limited cohort in association with the surface epithelium (Fraune and Bosch, 2007). Notable exceptions to these trends do occur; termites and their relatives, the cockroaches, appear to have several hundred phylotypes of bacteria (Cruden and Markovetz 1987; Schmitt-Wagner et al. 2003; Yang et al. 2005), as do certain sponge species (Grozdanov and Hentschel 2007). Whereas microarray analyses have been under way for several years in vertebrate symbiotic systems, they are only just beginning with the invertebrate–bacterial associations. The first such study involved a dual array of the pea-aphid host and its intracellular symbiont *Buchnera aphidicola* (Wilson et al. 2006).

The development of microarray tools for both vertebrate (see other sections of this chapter) and invertebrate aquaculture species (e.g., Chen et al. 2004; Jenny et al. 2007) paves the way for the study of host responses to beneficial bacterial partners. However,

as with any microarray study, acquisition of robust, informative data will require very careful design of the program of study, from the conception of the experimental setup to the interpretation of the resulting microarray data (see Implications for Aquaculture section).

A Case Study—Application of Microarray Technology to the Squid–Vibrio Symbiosis

The light-organ symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* has been studied for nearly 20 years as a model of beneficial host–bacterial interactions (for reviews, see Nyholm and McFall-Ngai 2004; Visick and Ruby 2006). The symbiosis has several features that render it a useful model system. *V. fischeri* is acquired each generation by the host and resides extracellularly along the apical surfaces of epithelia; thus, it engages in the most common type of symbiosis that animals have with bacteria. The host is relatively easily acquired from the field; it is a night-active predator along the shallow sand flats in the back reefs of the Hawaiian archipelago. The host provides the symbionts with nutrients for their growth and, in return, the symbionts provide the host with luminescence, which the host uses in its behavior. Thus, the "currency" of the symbiosis is not based on nutrition, i.e., the symbionts do not provide vitamins or amino acids as they do in most other animal–bacterial associations. For this reason, the host can be raised under laboratory conditions in the absence of the symbiont without apparent negative effects on host physiology. The symbiosis develops within hours of host hatching, and the onset of symbiosis can be assessed noninvasively by measuring host luminescence output with a sensitive photometer. Finally, genetic approaches have been well developed for the bacterial partner.

Early studies of this system focused on defining the anatomical, cellular, and biochemical events surrounding the onset, development, and maintenance of the association. These studies revealed a striking reciprocal dialogue between the partners, beginning in the first hours following exposure of the host to the symbiont (Figure 4.4a). This dialogue ensures that the association is specific, i.e., only *V. fischeri* colonizes host tissues. Analyses of the very first interactions between the partners, i.e., as they gather in host-shed mucus before entering host tissues, demonstrated that the specific symbiont is recognized by the host immediately upon their first interactions. Once the bacteria migrate into host tissues, a conspicuous symbiont-induced morphogenesis of the host organ ensues. Dramatic tissue remodeling transforms the light organ from a morphology that promotes colonization by the symbiont to one that mediates the mature function of the organ as a tissue that modulates the bacterial light for the host's behavioral uses.

Associated with each milestone in the onset of the symbiosis are particular cellular and biochemical events. Interestingly, both bacterial signal molecules (e.g., LPS and peptidoglycan derivatives) and host responses (e.g., cell swelling, cell death, macrophage-like cell migration, and cellular edema) are signals and responses that have typically been ascribed to pathogenic interactions. Thus, as beneficial interactions are turning out to be more common than realized, biologists may need to broaden their view of these types of cellular and biochemical "behaviors."

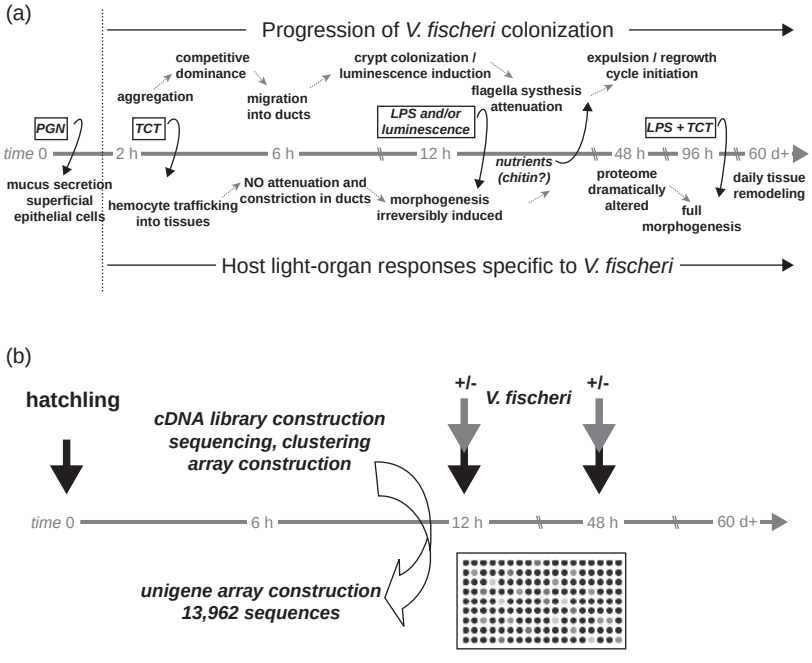

Figure 4.4. Strategy for characterizing symbiont-induced changes in the host transcriptome in the squid–vibrio symbiosis. (a) Studies of the activities of both partners of the association have revealed the complexity of the phenotypes underlying the host–symbiont interaction during early development (bacterial–symbiont activities, above the time line; host activities, below). Upon hatching, the host responds to the cell wall molecule (peptidoglycan, PGN) of nonspecific environmental bacteria by shedding the mucus in which the symbiont cells will aggregate. Specific host responses to *V. fischeri* begin around 2 hours following exposure. Bacterial signals known to induce changes in the host are boxed. LPS, lipopolysaccharide; NO, nitric oxide; TCT, "tracheal cytotoxin," monomeric peptidoglycan. (b) Genomic tools were created for the host squid with the intent of maximizing the number of transcripts expressed in light-organ tissues in response to the onset and early development of the association. A series of libraries were constructed at time points determined, by proteomic and anatomical analyses described above, to be critical developmental milestones in early symbiosis, specifically at hatching, and at 12 and 48 hours following hatching. At the later two times, libraries were made from animals that had been (+) and had not been (−) colonized. At 12 hours, in colonized animals, all conspicuous symbiont-induced developmental changes have been triggered; at 48 hours, the proteome of the symbiotic host is dramatically altered. A total of 11 libraries, including ones that were subtracted and normalized, were generated from mRNA isolated from these times. Following extensive sequencing and clustering of resulting sequences, a set of unique transcripts was identified. A representative clone from each cluster was selected for inclusion on the glass slide microarray. The microarray is being used to decipher the genomic events surrounding the onset, development, and maintenance of the symbiosis.

In addition to the processes of recognition, specificity, and development, symbiotic associations must achieve balance so that they are relatively stable over the life of the host; i.e., the symbiont cannot overgrow the host, nor can the host eliminate the symbiont with antimicrobial defenses. The squid–vibrio system achieves a dynamic balance of its symbiosis through a profound diel rhythm. At dawn each day, as the

host buries in the sand for its diurnal quiescent period, it vents 90–95% of its bacterial culture into the surround seawater, knocking back the population in the light organ. The culture then grows back up to fill the organ by the evening, when the host becomes active.

In analysis of the squid–vibrio symbiosis, it became apparent that it is a system in which biologists could define at the genomic level the minute-to-minute and hour-to-hour events in the establishment and maintenance of a symbiosis. With this broad goal in mind, the molecular tools were developed. The full genome sequence for a light organ isolate of *V. fischeri* was obtained (Ruby et al. 2005), annotated, and arrayed on an Affymetrix chip. For the host, an EST database was constructed from symbiotic and nonsymbiotic juvenile light organs at time points that had been determined to be critical in the host–symbiont dialogue (Figure 4.4b; Chun et al. 2006). Clustering of the sequences resulted in a set of nearly 14,000 unique clusters. A representative clone from each cluster was spotted on a glass-slide microarray. The intent is to couple the experimental tractability of the squid–vibrio system with these technical resources to dissect the molecular dialogue characteristic of a symbiosis. These data will be used to identify conserved strategies for symbiosis across the animal kingdom, and to define critical differences between pathogenesis and mutualism. Most exciting is the opportunity to characterize *both* the host and the symbiont dialogue at any time, under any set of conditions. In addition, the use of mutants in *V. fischeri*, particularly those that induce conspicuous phenotypes in the host, offers the opportunity to manipulate the partner dialogue experimentally. A current goal is to develop genetic approaches in the host so that its responses can also be manipulated at the molecular level.

Implications for Aquaculture

As in biomedicine, aquaculture has focused almost entirely on the relationships that aquaculture species have with pathogens. However, in efforts to improve aquaculture conditions, it may be worthwhile to define the normal, coevolved associations of cultured species with their beneficial bacteria, and the extent to which such associations are critical to health. As beneficial associations with bacteria are proving to be important for health in model vertebrates, such as zebrafish and mouse, it is likely that other vertebrates, such as those exploited in aquaculture, will also require such interactions. In approaching this area, several issues should be considered, including (1) Who are the members of the normal microbiota? Currently, researchers studying the vertebrate model systems are struggling with the complexity of this question. Microbes will grow in any nutrient-rich environment, so the core microbiota cannot be defined by simply enumerating the phylotypes within the tissues, i.e., one must define which constituents are coevolved partners and which are tourists (in the parlance of symbiosis, which are mutualists and which are commensals) and what is the interaction between these two groups. In addition, it may be important to study the normal microbiota in field-caught animals to determine the composition under those conditions in which the interactions have evolved. Culture conditions may drastically alter the microbiota, compromising health and growth of the animal. (2) What are the host responses to their microbiota, and how are these altered under various aquaculture conditions? Using such techniques as microarray analyses, we will be able to define changes of the host tissue responses between generations (e.g., the influence of increasing numbers

of generations away from the wild), as well as through the embryonic, larval, juvenile, and adult progression within a generation.

Although coevolved bacterial partners have been the focus of this discussion, other considerations may be highly relevant. For example, what about other microbes such as viruses and fungi? These abundant groups are likely to have at least as strong an impact as the bacteria. In addition, what are the relationships of aquaculture species to environmental microbes that are not coevolved partners? Most aquatic animals live in microbe-rich environments, where 10^5–10^6 bacteria per milliliter of water are bathing their tissues. Taken together, this frontier is extremely complex, but we now have molecular tools such as microarrays to ask how the microbial world interfaces with the world of animals to promote host health. The community of biologists is presented with an expansive and exciting horizon.

Future Directions

DNA microarrays for aquaculture-related research have developed rapidly over approximately the past 6 years, from platforms containing features representing relatively small numbers of genes (e.g., hundreds to a few thousand) to the latest platforms containing tens of thousands of unique features. For species of interest to the aquaculture industry, microarray platforms for global gene expression studies will likely continue to evolve to where they represent complete coverage of transcriptomes (e.g., oligo microarrays including unique features for all members of gene families and all splice variants). Additional microarray-based technologies (e.g., SNP chips for identifying sequence variants associated with production-relevant traits; chips for studying fish gut microbial communities; and new tools for studying host–pathogen and host–symbiont interactions) will also likely be developed and have positive influences on the future of aquaculture research.

In order to have completely characterized fish and shellfish transcriptomes, new genomic resources will be required. For example, targeted cDNA libraries (e.g., SSH and normalized) from specific tissues, life stages, and experimental stimulations (e.g., exposures to pathogens, parasites, and experimental stressors) must be created and characterized to fill in the gaps in current EST sets. These gene discovery efforts will be aided by genome sequencing projects for aquaculture-relevant species (e.g., the internationally supported project currently underway for Atlantic salmon). With the advent of massive throughput, lower cost DNA sequencing technologies, it is hoped that draft genome sequences will eventually be generated for many species important to global aquaculture. In addition to improving transcriptomic research, the existence of draft genome sequences will vastly improve our ability to conduct research in other areas such as proteomics and molecular evolution.

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Chapter 5
Aquaculture Genomics

Yniv Palti

What Is "Genomics"?

Genomics is the study of the genome. The term *genome* refers to the entire genetic content of an organism. In most cases, the genetic information is contained in deoxyribonucleic acid (DNA) molecules and as described in the central dogma theory of Francis Crick (1958) the DNA sequence is transcribed into ribonucleic acid (RNA) molecules and then translated from RNA into proteins. The entire RNA and protein content of an organism are referred to as transcriptome and proteome, respectively. Genomics, in the broad sense, includes transcriptomics (study of the transcriptome) and proteomics (study of the proteome), as the genetic signal can be modified during and after the transcription and translation processes. However, the technologies for studying the expression of genes or "functional genomics" are described elsewhere in this book (Chapters 3 and 4), and this chapter focuses on technologies used to study the genetic information stored in the DNA of cultured finfish.

The genomes of all eukaryotic cells are organized in chromosomes localized to the nucleus compartment of the cell. A very small portion of the genome of animals and plants is in a DNA molecule enclosed within mitochondrial compartments of each cell, and plants also have DNA in their chloroplasts. With very few exceptions, each cell of an organism has the exact same DNA blueprint. However, the large variation of cell types, tissues, developmental stages, and physiological conditions exhibited in each individual is caused by the differential expression of genes, which determines cell types and developmental stages.

In most cases, the number of chromosome pairs of an individual species is fixed, that is to say, that all individual members of that species have the same karyotype or chromosome morphology. One obvious exception is the sex chromosomes where often the male chromosome Y is smaller than the X chromosome. In rainbow trout, the number of chromosome pairs (n) can vary from 29 to 32 and is thought to be affected by geographic distribution of the original population (Thorgaard 1983). Still, even in rainbow trout the number of chromosome arms is fixed across all individuals. The number of chromosome arms is determined by the location of the centromere. A chromosome is considered to have two arms when the centromere (Figure 5.1) is in the middle and one arm when it is terminal.

The germ cells are said to be haploid with n chromosomes. The fusion of the sperm and egg nuclei postfertilization gives rise to a diploid cell state with $2n$ chromosomes. During the process of meiosis that produces eggs and sperm, the homologous chromosomes randomly segregate to produce single chromosome sets (n). The variation in the number of chromosomes between species is very large. Humans, for example, have 46 chromosomes ($4n = 23$). In salmonids, it is possible to artificially induce

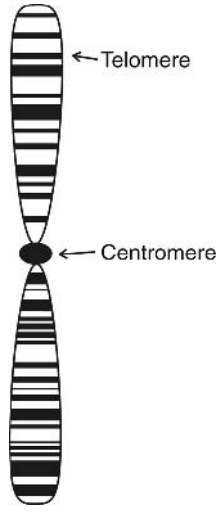

Figure 5.1. Chromosome showing the locations of the centromere and telomere.

tetraploidy where the somatic cells have 4*n* chromosomes and the germ cells have 2*n* chromosomes (Hershberger and Hostuttler 2005). Methods regarding chromosomal manipulation are covered in Chapter 8. The total length of the genome of an organism is composed of the sum of all the chromosomes in the cell. The approximate size of the genome is measured by weight; 1 pg of DNA is approximately 978 million base pairs (Mbp). There is more than tenfold variation in genome size within teleosts ranging from 0.4 to more than 4.0 pg. Here are some estimates of the genome size of the most widely cultivated fishes taken from the Animal Genome Database (Gregory 2005; http://www.genomesize.com/ and references therein): catfish (1.0 pg), tilapia (1.0 pg), common carp (1.8 pg), Atlantic salmon (3.0 pg), rainbow trout (2.8 pg), striped sea bass (0.9 pg), Atlantic cod (0.9 pg), and gilthead sea bream (0.95 pg). Knowledge of the genome size is important for preparing tools and reagents for a species. There is a direct relationship between the size of the genome and the amount of money, time, and work that are needed to be invested in obtaining sufficient genome coverage for the genetic map, physical map, and ultimately a whole-genome sequence.

The number of genes in a genome is another parameter for assessing genome complexity, but it may not be linearly correlated to the biological complexity of the organism. The number of unique proteins is probably a better indicator of biological complexity, as more than one unique protein can be produced from a single gene or open reading frame (ORF) via alternative splicing, posttranscriptional modification, and posttranslational modifications. Here are some numbers that provide insight on the relationship between the number of genes and the biological complexity of the organism. The number of genes in the genome of the bacteria *Escherichia coli* is just more than 4,000 (Blattner et al. 1997), while it was estimated that the relatively simple roundworm *Caenorhabditis elegans* has more than 19,000 genes (*C. elegans* Genome Sequencing Consortium 1998) and the human genome contains fewer than 30,000 genes (Human Genome Sequencing Consortium 2001; Venter et al. 2001). As a result of whole-genome duplications that occurred during teleost evolution (Allendorf and Thorgaard 1984; Amores et al. 1998; Jaillon et al. 2004), it is likely that the number of

genes described in the genomes of commercially reared fish will be higher than that for the human genome. Although genes coding for protein are probably the most studied aspect of the genome, most vertebrate genomes contain large amounts of noncoding DNA that is not transcribed into RNA. Furthermore, large fractions of this noncoding DNA of the genome are composed of repetitive sequences that increase the difficulty in the assembly and annotation of the genome sequence. Therefore, it is expected that the larger fish genomes will have larger fractions of noncoding repetitive sequences and will be harder to assemble and annotate.

Genomics has revolutionized the way research is done in the medical field as well as in plant and livestock agriculture. The ultimate goals of genomics research in aquaculture are to (1) understand how the genetic makeup affects the biology of the organism and (2) develop diagnostic tools to predict performance based on the genes and genome content of each individual. Genomics has a very broad scope aiming to look at all the genes of an organism and how they interact and affect biological processes and pathways. It requires large-scale development of species-specific tools and reagents. However, the development of research reagents is meaningless without the ability of linking the genotype and phenotype of the organism, which requires a multidisciplinary research approach and close collaboration between molecular and quantitative geneticists, physiologists, immunologists, microbiologists, and so on. The large volume of data generated by genomics research requires a high-throughput pipeline for data processing and analysis, including sophisticated mathematical algorithms and statistical tools. This new interface of computer scientists, statisticians, mathematicians, and biologists created the new scientific discipline called "Bioinformatics."

Genome Mapping

The ultimate genome map is the actual DNA sequence, base pair by base pair, for all the species' chromosomes. However, the cost of whole-genome sequencing utilizing current technologies is very high and requires the availability of very dense genetic and physical maps to aid in the correct assembly and ordering of the DNA sequence fragments along the chromosomes. A combination of deep coverage genetic and physical maps provides very powerful research tools that can be used for identifying economically important quantitative trait loci (QTL) and for fine mapping and isolating a specific gene or genes affecting the trait of interest.

DNA Marker Technology

The onset of every genome mapping project is the development of genetic markers to be used as landmarks along the chromosomes. Genetic markers are useful if they can capture the allelic variation (polymorphism) between individuals and between the two homologous copies that are inherited from the parents of each individual. Certain phenotypes (e.g., sex) can be used as markers, and in the past protein allozymes were used as markers in aquaculture genetics research. However, the challenge of genomics research is that a very large number of single-locus polymorphic markers (more than 1,000 for species with large genome such as the salmonids) are needed to enable taking a snapshot of each individual genome. Furthermore, new genotyping platforms

for genome-wide association (GWA) studies utilize from 60,000 markers (Bovine) to 500,000 markers (Human) in a single reaction, and are currently used in medical and livestock agricultural research. The current state of the art in DNA marker technology enables robust pipelines for rapid development, screening, and genotyping of markers. Although these technologies require sophisticated and expensive equipment that is beyond the reach of most individual laboratories, it is important to understand the principles of these technologies and to be able to use them in aquaculture research through collaborations or outsourcing. Here, I will describe the types of DNA markers that are most widely used in current genomics research and how they are identified and developed into a standard assay. For a broader review of DNA markers technologies in aquaculture research, the readers are encouraged to read the review of Liu and Cordes (2004) or Chapters 2 through 9 in the book *Aquaculture Genome Technologies* (Liu 2007).

Markers Presently in Use

Microsatellites

Microsatellites are the most useful marker for genetic mapping. By far, they are the most polymorphic DNA marker. They are abundant throughout the genome of all vertebrates studied to date, and their codominant inheritance allows for scoring of all parental alleles (up to four) in the offspring. They are also very useful in the integration of the genetic map with the physical map and with the genome sequence because they are sequence tagged and can be isolated from expressed sequence tags (ESTs) and from end sequences of bacterial artificial chromosomes (BACs). Historically, they have been called microsatellites (Litt and Lutty 1989; Weber and May 1989), SSRs for simple sequence repeats (Tautz 1989), or STRs for short tandem repeats (Edwards et al. 1991), but microsatellite is the term adopted by most. Microsatellites are made up of tandem short sequence repeats (1–6 bp). The repeats are classified based on the tandem motif from mononucleotide for a single base pair (e.g., A_n) to hexanucleotide for 6 bp (e.g., $[ATGCAC]_n$). The core repeat can be composed of a simple repeat (e.g., $[CA]_{10}$), incomplete repeat (e.g., $[CA]_{10}G[CA]_6$), or a compound repeat (e.g., $[CA]_8[ATC]_6$). The genetic marker is made of two unique polymerase chain reaction (PCR) primers that flank the core repeat and produce an amplicon fragment in the size range of 80–400 bp. Allelic variation is detected by high-resolution electrophoretic separation of the fragments. When first developed as genetic markers, the visualization of the fragments was done by radioactive labeling or silver staining of the fragments in denaturing polyacrylamide gels, whereby several lanes per gel were dedicated for a size standard. Advancements in fluorescent labeling and the development of automated DNA sequencers coupled with software for assigning fragment size based on internal size standards for each sample now enable streamlined and robust genotyping of microsatellites.

Polymorphism

Microsatellite polymorphism is based on allele size differences that are most often caused by differences in the number of core repeats. In humans, estimate of the

mutation rate in microsatellite repeat regions is approximately 10^{-3} per locus per generation (Weber and Wong 1993), which is approximately six orders of magnitude higher than the estimated mutation rate of nonrepetitive DNA. Two models were proposed to explain this high mutation rate: the first involves slipped strand mispairing during DNA replication (Levinson and Gutman 1987) and the second involves nonreciprocal recombination between two microsatellites (Jeffreys et al. 1994). Resultant of the first model is variation within the microsatellite of one or two repeats, while in the latter model the results will likely produce alleles with large size differences. Typically, microsatellites with large number of repeats are more polymorphic, which is in line with both models.

Abundance

Microsatellites are highly abundant in all aquaculture species studied to date. For example, 3.21% of the compact genome of the puffer fish *Tetraodon nigroviridis* is composed of microsatellites (Crollius et al. 2000). In catfish, it was estimated from BAC end sequences (BES) that 2.58% of the genome is composed of microsatellites (excluding mononucleotide repeats), and a microsatellite is found every 2.67 kilobase (kb) (Xu et al. 2006). In the human genome, for comparison, a microsatellite is found approximately every 6 kb (Beckmann and Weber 1992). Mononucleotide repeats are the most abundant form of microsatellite, but they are not suitable for use as markers. Consistent allele sizing for mononucleotides is nearly impossible even with the most advanced technology due to certain attributes of PCR kinetics. Therefore, the dinucleotides are the most abundant form used as genetic markers followed by trinucleotides and tetranucleotides.

Genomic Distribution

Microsatellites have been found to be evenly distributed throughout the genomes of all teleosts studied to date (Naruse et al. 2000; Woods et al. 2000; Waldbieser et al. 2001; Kai et al. 2005; Guyomard et al. 2006). They are found in much higher frequency in noncoding regions of the genome, possibly due to negative selection against frame-shift mutations in the coding sequences (Metzgar et al. 2000). Nonetheless, large sequencing projects of transcribed DNA in salmonids and catfish revealed that microsatellites are also abundant in coding regions (Serapion et al. 2004; Coulibaly et al. 2005; Ng et al. 2005; Rexroad et al. 2005).

Inheritance

Microsatellites are inherited in a codominant Mendelian fashion, which enables scoring of all alleles in each individual (two alleles per locus in heterozygotes and one allele in homozygotes). This feature makes them extremely useful for genetic mapping. One caveat is the presence of null alleles in some microsatellites due to mismatch of the PCR primers or insertion/deletion within the amplified fragment. The presence of null alleles can cause genotyping errors, where a heterozygous for an amplified/detectable allele and a null allele can be scored as a homozygous for the detectable allele (Johnson et al. 2007). They are found in unusual high frequency in salmonids (e.g., Morris et al. 1996). Typically, in genetic mapping projects where pedigree is known

and all parents and offspring are genotyped, null alleles can be identified and are still useful for QTL detection. However, in population association studies they are much harder to detect, and caution should be exercised in the selection of markers for the study (e.g., Johnson et al. 2008).

Development of Microsatellite Markers

Construction of small insert genomic libraries enriched for microsatellites has been the most common approach for robust isolation of microsatellites in new genome projects (Ostrander et al. 1992; Rexroad et al. 2002). However, these are markers from anonymous regions of the genome that for the most part are noncoding (type II markers). Type I markers that are part of the coding sequence of a gene are useful for comparative genome analyses with sequenced genomes of model organisms and also for predicting which genes are linked to the markers. In more advanced genome projects that have large EST databases and BAC libraries, microsatellites can be detected in the EST database by specialized software and developed to be type I markers (e.g., Serapion et al. 2004; Rexroad et al. 2005). The caveat here is that most genes of interest for physiological and comparative studies are not likely to have microsatellites in their coding sequence. Several methods for PCR screening and partial sequencing of BACs that harbor genes of interest have been developed and used in aquaculture genomics research (Waldbieser et al. 2003; Rodriguez et al. 2006). These methods have been successful for developing microsatellite markers tightly linked to the genes of interest, but they are very low throughput and do not generate markers fast enough for large mapping projects. Another excellent source for robust development of microsatellites comes from BES projects. In catfish, 17.5% of approximately 20,000 BES contained microsatellites (Xu et al. 2006), and in Atlantic salmon approximately 5% of 200,000 BES contained microsatellites with sufficient flanking sequence for designing PCR primers (William Davidson, personal communication). Markers isolated from BACs can be used for integration of the BAC physical map with the genetic map in addition to the "traditional" role of increasing the density of the genetic map (e.g., Somridhivej et al. 2008).

Amplified Fragment Length Polymorphism

The technique was first described by Vos et al. (1995) and was quickly adapted for genetic linkage mapping and QTL identification in aquaculture genomics research (e.g., Kocher et al. 1998; Young et al. 1998; Liu et al. 1999, 2003; Agresti et al. 2000; Robison et al. 2001). Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique that does not require prior sequence knowledge or genetic information on the species. It is more robust, reliable, and efficient than other fingerprinting methods used in aquaculture genomics such as multilocus restriction fragment length polymorphism (RFLP, see below) and random amplified polymorphic DNA (RAPD, see below). AFLP markers are inherited in a dominant Mendelian fashion. The technique enables simultaneous screening of many loci and typically more than 100 markers can be genotyped in a single PCR.

Molecular Basis of the Technique

AFLP combines the reproducibility of restriction enzyme digestion with the robustness of PCR. Restriction enzymes or restriction endonucleases are bacterial enzymes that recognize and cleave the DNA in a specific 4–8 bp long sequence motif. In the first step, the genomic DNA is digested using two restriction enzymes. The cutting frequency of the genome by a restriction enzyme is mainly a function of two parameters. (1) The length of the recognition sequence: A 4-bp recognition sequence will occur on average every 256 bp (4^4) and therefore at much higher frequency than a 6-bp sequence that will occur in the genome on average every 4,096 bp (4^6). (2) The actual base content of the genome: A genome with unusually high AT content will provide higher frequency of restriction sites for enzymes that use AT-rich recognition sequence. Typically in fish a six-cutter (e.g., EcoRI) and a four-cutter (e.g., MseI) are used in the first step. In a genome of 1×10^9 bp (catfish or tilapia), this double-enzyme restriction digestion is expected to produce 500,000 EcoRI–MseI fragments and approximately 1,500,000 EcoRI–MseI fragments in a salmonid genome. In the second step, double-stranded adaptors of known sequence are ligated to the fragment ends. The adaptors match the overhang sequence as cut by the restriction enzyme, which allows using a different sequence for the EcoRI end adaptor and for the MseI adaptor. In the third step, a subset of the fragments is amplified by PCR. The primers used are composed of the sequence of the EcoRI or MseI adaptor plus an arbitrary base on the 3′ end. In the fourth step, the amplified subset from the third step is amplified by another round of PCR, but this time three arbitrary bases are added to the 3′ end of the primers (the same base that was added in the third step and another two bases). This selective PCR is expected to reduce the number of detectable fragments. As only the EcoRI+3 primers in the fourth step are labeled, the expected number of fragments resolved and detected by gel electrophoresis is between 125 for catfish or tilapia and 375 for trout or salmon, but in practice the number of resolved fragments is much smaller than that.

AFLP Polymorphism

Changes in the three bases adjacent to the restriction enzyme recognition site can be detected by the appearance or disappearance of AFLP bands. Additionally, sequence variation causing changes in the restriction enzyme recognition site as well as deletions or insertions between any EcoRI and MseI recognition sites will lead to changes in fragment size and polymorphism between individuals. As AFLP screens large number of loci in a single reaction, the method detects enormous genetic variation.

Inheritance of AFLP Markers

AFLP markers are inherited as dominant markers, meaning that the homozygous and heterozygous genotypes cannot be distinguished. In dominant markers only one allele is scored per fragment, and the alternative allele is scored as another locus. As a result, the number of loci is inflated and transfer of information across strains and even families within the same species is very difficult and impractical. In addition, if large size differences occur between the two alternative alleles, preferential PCR

amplification of smaller targets may cause loss of the larger alternative allele in the heterozygotes altogether.

Strengths and Weaknesses

The major strengths of AFLP are that the technique does not require prior DNA sequence and molecular information on the studied species; it generates a large number of polymorphic markers and genotypes a large number of loci, all at a relatively low cost per locus and marker. It is more reliable than RAPD, the other PCR-based DNA fingerprinting method, and more robust than RFLP. These advantages made AFLP useful for population genetics studies in fish (e.g., Campbell and Bernatchez 2004; Simmons et al. 2006) and for initial genetic maps and QTL detection studies in aquaculture (e.g., Kocher et al. 1998; Young et al. 1998; Liu et al. 1999, 2003; Agresti et al. 2000; Robison et al. 2001). The major weaknesses are the dominant inheritance imposing enormous difficulties on the transfer of information and the additional laborious steps that are required in developing single-locus markers from informative polymorphic AFLP fragments (e.g., Felip et al. 2004).

Markers of the Future

Single-Nucleotide Polymorphisms

Single-nucleotide polymorphisms (SNPs) are caused by point mutations at a specific nucleotide position, giving rise to alternative bases at this location. For the past three decades, they have been known to be the ultimate source of genetic variation and the most abundant polymorphism in any organism. Like microsatellites, they have codominant Mendelian inheritance, but per marker they are much less polymorphic than microsatellites. In theory, an SNP can have as many as four alleles (A, T, C, or G), but in practice they are biallelic (Vignal et al. 2002). As genetic markers, SNPs have two major advantages over microsatellites: (1) they are much more abundant in the genome (Sachidanandam et al. 2001; Hirschhorn and Daly 2005) and (2) they are more adaptable to genotype automation (Lai 2001).

A major lesson from genetic studies of multifactorial human diseases was that a very high-density SNP map is needed for screening of every linkage disequilibrium block in the human genome (Hirschhorn and Daly 2005; Altshuler and Daly 2007). With the completion of the human genome sequence at the turn of the millennium (Altshuler et al. 2000; Human Genome Sequencing Consortium 2001; Venter et al. 2001), it became clear that the new challenge in human genomics was resequencing of individual genomes for SNP discovery and the development of genotyping platforms for high throughput and more affordable whole-genome assays. A concentrated resequencing effort and the development of gene chip technology lead to a recent burst of GWA studies for complex diseases that used a 500,000 SNPs chip for the very precise identification of loci affecting disease onset (Altshuler and Daly 2007; The Wellcome Trust Case Control Consortium 2007). It is likely that a very large number of SNP markers will be necessary for accurate and rapid identification of loci affecting complex quantitative traits in livestock and aquaculture (The International Chicken Polymorphism Map Consortium 2004; Lindblad-Toh et al. 2005; Aerts et al. 2007;

Khatkar et al. 2007). New DNA sequencing and robust SNP genotyping technologies developed for the human genome project can be adapted for the discovery and genotyping of large numbers of SNPs for aquaculture genomics research. The large number of new markers can dramatically increase the density of genetic maps and the efficiency of identifying loci that affect economically important complex traits in aquaculture. Still, in the absence of genome sequence, a major challenge for aquaculture genomics research will be to validate the tens of thousands of putative new SNPs.

SNP Discovery

Data mining of a large sequence database and the alignment of sequences of the same locus from different individuals and from the two haploid alleles of heterozygous individuals are at the core of any robust SNP discovery approach. The pool of individuals often representing several breeds or populations of economic and scientific interest is termed the SNP discovery panel. For the aquaculture species that already have an extensive EST database, it appears this could be a good resource for SNP discovery if the EST libraries are made from several outbred individuals (Hayes et al. 2007). A major strength of this approach is that all the SNPs identified can be developed into type I markers. However, this is also a limitation for this approach because evolutionary selection is much stronger against mutations in the coding regions, and therefore fewer SNPs are expected to exist in the ESTs compared to noncoding regions. Another major limitation with teleosts is the high number of paralogs in their genome as a result of the whole-genome duplication of their common ancestor (Amores et al. 1998; Jaillon et al. 2004). These paralogous genes still share a large portion of their coding sequence, and this results in many of the base sites that appear to be SNPs in the alignment of highly similar sequences to be false SNPs resulting from sequence differences between the paralogs. This problem is even more severe in the salmonids that experienced an additional and relatively recent whole-genome duplication of their common ancestor (Allendorf and Thorgaard 1984). Therefore, it is important to employ rigorous criteria in the SNP discovery pipeline and to use an extensive validation panel as described by Hayes et al. (2007). Recently, Moen et al. (2008) constructed the first EST SNPs-based genetic map for aquaculture species (Atlantic salmon) and similar projects are under way for rainbow trout and catfish.

Another approach that can be used for species that have a large database of BES is to identify unique sequences in this database and design PCR primers for the production of amplicons from a representative panel of individuals. The amplicons of all the different loci from each individual on the SNP discovery panel can then be pooled together and used as a template for pyrosequencing with one of the new DNA sequencing technologies (Bentley 2006; Huse et al. 2007; Van Tassell et al. 2008). The new sequencing technologies provide a very large number of single-allele (or haploid) sequences that offer a very good representation of all the loci and their alleles present in the pooled samples at a very low cost per sequence. However, this method requires very expensive and specialized equipment. For the discrimination between allelic variation and false paralogous SNPs, it is important to include in the discovery panel clonal homozygous individuals produced by androgenesis or gynogenesis or DNA from individual haploid embryos (Young et al. 1996; Palti et al. 1997a). Another issue

that should be considered in all SNP discovery projects that use new pyrosequencing technologies is the additional investment in computer storage and processing capacity that will be required for analyzing the large amount of data (e.g., Trombetti et al. 2007). When a BAC physical map is available (see BAC libraries and physical maps section), this approach can be very useful for integrating the physical and genetic maps. A major drawback of this approach is that it requires a very large investment of labor, time, and reagents for generating a very large number of locus-specific PCR amplicons.

A third approach is to simply random sequence, using multiple-fold coverage, each individual in the SNP discovery panel via random shearing of each individual genome and subcloning of the sheared fragments into shotgun libraries (The International Chicken Polymorphism Map Consortium 2004). The drawback here is the large effort of making high-quality shotgun libraries and the enormous amount of sequencing needed to obtain severalfold genome coverage of each individual from the discovery panel.

A fourth approach is based on the reduced representation shotgun sequencing (RSS) method of Altshuler et al. (2000). In this approach, the genome samples from the discovery panel individuals are cleaved with specific restriction enzymes and separated by agarose gel electrophoresis to enable size selection of the 600-bp fragments. To generate the database for SNP discovery, those fragments can be directly sequenced using one of the new pyrosequencing methods or subcloned and sequenced using the "traditional" Sanger sequencing method. The rational behind this approach is that by using specific restriction enzymes, genomic segments from the same origin will migrate to the same size range on the electrophoretic gel and will provide reduced representation of the discovery panel genomes. Clearly, this method in conjunction with the new pyrosequencing technologies provides answers to the weaknesses and drawbacks of the other three approaches (Van Tassell et al. 2008).

Genotyping Methods

Several "traditional" SNP genotyping methods that do not require extensive DNA sequencing and very expensive equipment have been previously used in aquaculture research. These very low-throughput methods may be useful for mapping or studying the genetic variation of a specific gene or locus with a relatively small number of samples.

Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (e.g., Palti et al. 2001) is based on the fact that a single base change can cause changes in the secondary structure of single-stranded DNA. In this method, a targeted locus is amplified by PCR and the double-stranded DNA is denatured and the self-annealing of the single strands takes place in conditions that favor single-strand conformations rather than annealing of the two strands. The products are then separated by high-resolution electrophoresis on a nondenaturing gel.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (e.g., Brunvold et al. 2007) is based on the fact that DNA fragments with lower GC content will denature faster. In this method, the PCR amplicon is electrophoresed through a polyacrylamide gel with denaturing gradients (temperature or pH). As the DNA samples progress through the increasingly stringent gradients, the double-stranded DNA is "melting," and the separation of the two strands slows down the movement of the DNA. Fragments with lower GC content will start melting earlier, and their movement across the gel will be slower. Therefore, the individual samples will be separated according to their DNA sequence.

Polymerase Chain Reaction–Restriction Fragment Length Polymorphism

RFLP or RSP (restriction site polymorphism; e.g., Hansen et al. 1999) is based on the presence of an SNP in a specific sequence motif recognized and cleaved by restriction enzymes. The sequence motif or restriction site can be composed of 4–8 bp. A change of a single base in the motif recognized by a specific enzyme will prevent it from cleaving the DNA. After treatment of the PCR amplicon with a restriction enzyme the samples are separated by agarose gel electrophoresis, and changes in the number of fragments and fragment size imply sequence variation between the samples.

Recent High Throughput and Robust SNP Genotyping Methods

The current state-of-the-art SNP genotyping technologies and platforms can be divided into two groups: (1) samples are genotyped one SNP at a time and (2) each sample is simultaneously genotyped for a large number of SNPs (up to one million in a single pass through) using the concept of the DNA-chip or gene-chip technology (see below and also in Chapter 4). Two very popular representatives of the first group are the TaqMan technology (e.g., Li et al. 2004) and matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Ross et al. 1998; Pusch et al. 2002). Recently, the gene-chip technology has become very popular in medical research through the use of two commercially available platforms. They are called the DNA glass-chip platform and the bead-array platform.

TaqMan

PCR primers that flank the SNP are designed along with a TaqMan probe for each allele (typically two). The TaqMan probes annealing temperature is higher than the PCR primers to enable their annealing to the DNA template prior to the primers. Each probe is labeled by two fluorescent dyes, a reporter and a quencher. Each SNP allele is represented by a specific probe that in turn is labeled with a different reporter dye. As the Taq DNA polymerase amplifies the PCR template, it also degrades the probe by its 5′–3′ exonuclease activity and releases the reporter dye from the close proximity and quenching activity of the other dye. The fluorescence intensity is detected and measured by the real-time PCR instrument (see Chapter 3). The probe that has the

perfect match to the sample DNA sequence is also degraded more efficiently by the Taq polymerase, and its fluorescent signal is detected by the instrument.

MALDI-TOF

As with the TaqMan approach, SNP genotyping with MALDI-TOF requires prior knowledge of the SNP sequence and involves prior amplification of that sequence by PCR. The post-PCR assay is based on annealing of a third primer exactly upstream of the known point mutation and extension of the primer with dideoxy nucleotides (ddNTPs). The most important property of ddNTPs is that they terminate the elongation of the primer's strand by the DNA polymerase (for more information on the properties of ddNTPs, see the section on Sanger DNA sequencing). Therefore, only one ddNTP (A, G, T, or C) complementing the sequence of the DNA sample can be added to the primer. Mass spectrometry is used to separate and detect the alleles based on the differences in molecular weight of the added nucleotide. The mass spectrometer instrument is very expensive (e.g., Sequenom's MassArray system), but the technology is robust with relatively low cost per genotype. This technology is routinely used in livestock genetics research (e.g., Rohrer et al. 2007).

DNA Chip Platform

The DNA chip approach is based on hybridization of a fluorescently labeled unknown sample of DNA or RNA to a large number of single-stranded DNA oligonucleotides (oligos) that are bound to a small glass slide in a specific order. The posthybridized slide is scanned by a high-resolution scanner that detects the "spots" of fluorescence emission. Those spots are compared to the known array of spotted oligos on the slide and reveal the identity of the sequences to which the unknown sample hybridized (see Chapter 4 for more details on this technology). For SNP detection, the spotted oligos represent exact matches to the known alleles of a very large number of SNPs (most recently 500,000 SNPs on one human chip; The Wellcome Trust Case Control Consortium 2007). To determine which alleles are present for each SNP, the labeled individual genomic DNA sample is hybridized to the slide under heat and salinity conditions that allow only selective annealing of the DNA molecules whose sequences are an exact match to the specific oligos (alleles) sequences. Prior to scanning, the slide is washed to remove the excess DNA molecules that did not anneal to the oligos on the slide. The DNA chip platforms allow for a very robust genotyping of a very large number of SNPs in a single pass through processing.

Bead-Array Platform

Recently developed by Illumina (Oliphant et al. 2002; Fan et al. 2003), the bead-array platform is more flexible and appears to be even higher throughput than the DNA chip platform. This platform is available in two assay systems, the flexible and popular GoldenGate assay and the more recent and high-throughput iSelect assay. Here is a brief description of the GoldenGate assay. Three oligos are used per SNP, the allele-specific P1 and P2 and the locus-specific P3. P1 and P2 are labeled by unique fluorescent dyes. P3 is composed of a nucleotide sequence adjacent to the SNP position. The three oligos are tailed by universal primers that are used for PCR

amplification of the unknown genomic DNA. After processing, the denatured PCR products are hybridized to the bead array. The sequence of P3 complements the oligo attached to a specific bead with a unique identifier for that particular SNP. P3 is now conjugated to either P1 or P2 via PCR (or both in heterzygotes). The posthybridized bead array is scanned by the BeadArray Reader, and the fluorescence signal from P1 and/or P2 at each unique bead site is analyzed by automated genotyping software. The bead-array platform requires very expensive equipment that is beyond the reach of individual aquaculture genomics research laboratories, but genotyping services are available from the company or through core facilities at universities and other research institutions.

Handling and processing of the extreme amount of data require specialized computer equipment and skills and the capacity to conduct very complex statistical analyses, in addition to the expensive equipment needed for the SNP chip platforms (Altshuler and Daly 2007; Trombetti et al. 2007). The robust and automated SNP marker technology has a great promise for aquaculture genomics research, but it is likely that the application of the technology will occur by collaborative efforts with few central laboratories and dissemination of the data back to the individual researchers for interpretation of the biological and aquaculture relevance.

Markers of the Past

Restriction Fragment Length Polymorphism

Single-locus RFLP was the marker of choice in the early days of genomics (Soller et al. 1976; Botstein et al. 1980). This method combined two technological breakthroughs from the 1970s: the discovery of restriction enzymes (Sambrook et al. 1989 and references therein) and the development of the Southern blot analysis (Southern 1975). When aquaculture finally joined the genomics era in the 1990s, RFLP was mostly used in its multilocus DNA fingerprinting form (e.g., Palti et al. 1997b, 1999) or some variation of PCR-RFLP (e.g., Hansen et al. 1997, 1999).

Molecular Basis

RFLP is based on resolving length differences of DNA fragments from restriction enzyme digestion using gel electrophoresis. Traditionally, genomic DNA was digested with the specific enzyme of choice and resolved overnight at low voltage and temperature on 0.7–1% agarose gel. Then the DNA was transferred by capillary action or by using an electromagnetic field with a nitrocellulose or nylon membrane and cross-linking to the solid support membrane (e.g., by brief exposure to UV light). The membrane can then be hybridized to a locus-specific probe made of a labeled nucleic acid molecule representing the sequence of the locus, or to a multilocus probe composed of some type of repetitive DNA sequence (Jeffreys et al. 1985). The probes were typically labeled by ^{32}P radioisotope, but can also be conjugated to alkaline phosphatase for chemiluminescence detection or fluorescently labeled. The DNA bands can then be visualized by exposing X-ray film to the probed membranes or by digital scanning of the membranes. The other common use of the method is in PCR-RFLP or RSP as described above under traditional SNP genotyping methods.

Polymorphism

RFLP polymorphism is caused by changes to the restriction enzyme recognition site or by major insertion/deletion events leading to significant restriction fragment size differences. The polymorphism of single-locus (SL)-RFLP markers is very low compared with microsatellites. Multilocus (ML)-RFLPs are more polymorphic than SL-RFLP simply because they detect a larger number of loci in a single hybridization, but they are not as robust as AFLP.

Inheritance

Like microsatellites, SL-RFLPs are inherited as codominant markers, where both alleles are detected in the heterozygote. ML-RFLPs suffer from the same problem affecting other DNA fingerprinting methods, where the alternative allele is likely to be scored as another locus. This is caused partly because of the large number of fragments per sample and partly because it is very difficult to resolve and differentiate small fragments (typically <2 kb) on the large gels used for ML-RFLP.

Strengths and Weaknesses

A major advantage of SL-RFLP is that they are codominant markers. However, they are much less polymorphic than microsatellites and compared to SNPs they are much less abundant in the genome and less adaptable for automated genotyping. The advantage of ML-RFLP for aquaculture species is that they require very little prior sequence information, but they are harder to score and reveal much less sequence variation than AFLP. In current aquaculture genome research, RFLP gave way to microsatellites and AFLP, but PCR-RFLP is still used for revealing sequence variation in specific genes of interest.

Random Amplified Polymorphic DNA

RAPD is a PCR-based DNA fingerprinting technique. The method was first described in 1990 (Welsh and McClelland 1990; Williams et al. 1990) and was fairly popular in species with low or no sequence information throughout the 1990s. It is by far less reliable and less informative than AFLP, but the procedure is relatively simple and the required equipment and technical skills are minimal.

Molecular Basis

A single short primer (8–10 bp) is used in a PCR with low annealing temperature (36–44°C) to amplify anonymous fragments of genomic DNA. Arbitrary primer sequences are used, and amplification occurs where the random short primer sequence is found in opposite direction on the two DNA strands at a distance short enough to enable PCR amplification (up to several thousand base pairs). Typically, 5–20 PCR fragments are produced and resolved using agarose gel electrophoresis from a single-primer reaction. The banding patterns of individual samples are compared, and presence/absence of individual bands is scored as polymorphism.

Polymorphism

RAPD polymorphism is caused by base substitutions, deletions, or any other changes in the sequence at the RAPD primer binding sites that can lead to loss or gain of bands. Additionally, major insertion/deletion events between any two primer binding sites can cause changes in the size of an existing band as well as loss or gain of a band. Like other DNA fingerprinting methods, relatively high levels of polymorphism can be observed from a single RAPD reaction, because several loci are being scored in one shot. However, the number of bands produced by a single RAPD reaction is smaller than ML-RFLP and AFLP.

Inheritance

As described above for the other two fingerprinting methods, RAPDs are inherited and scored as dominant markers. It is almost impossible to differentiate between homozygote and heterozygote bands, and it is impossible to determine if band size differences are caused by locus or allelic variation.

Strengths and Weaknesses

The major strengths of RAPD are that prior knowledge of genome sequence is not needed and the equipment necessary to perform the assay is relatively simple and inexpensive. The major weaknesses, in addition to the inherited problems of DNA fingerprinting methods, are low reproducibility caused by the low- and less-specific primer annealing temperature and lower polymorphism that typically is not sufficient to detect genetic differences between individuals from the same population or family. These weaknesses have limited the use of RAPD in aquaculture genomics, and in recent years this method was pushed aside by AFLP and microsatellites.

Genetic Mapping

Genetic linkage maps are the most common tool used in genomics research to provide first approximation of chromosomal organization. Genetic markers are arrayed on linkage groups (LGs) that represent entire chromosomes or portions of chromosomes. The first requirement for mapping a marker is that it needs to be heterozygous in at least one of the parents. In addition, pedigree information must be known for at least one generation, and a large data set of progeny genotypes must be available to produce a mapping panel. As all aquaculture species considered in this chapter have the ability to generate large numbers of progeny, we assume that full-sib families with a large number of progeny are available for genotyping and construction of a genetic map.

Genetic mapping is based on the Mendelian concept of independent assortment of markers and genes. The segregation of marker alleles is expected to be random, unless the two markers are physically linked along the same chromosome. The construction of a linear map is a process composed of three types of calculations. First, the two-point recombination distances are calculated to identify all the linked marker pairs and established LGs. Second, the ordering of the markers is accomplished by calculating

the likelihood of all possible orders along the linear chromosome. Third, the genetic distance between the markers and along the chromosome is calculated.

The distance between markers along the chromosome is measured by the rate of meiotic recombination events or cross-overs occurring between nonsister chromatids. Typically, the allelic combination of markers A and B found in high frequency is assumed to be the parental phase, and the low-frequency combination is assumed to be the recombinant. The recombination fraction theta (θ) is calculated as:

$$\theta = \text{recombinants}/(\text{parentals} + \text{recombinants})$$

A θ of 50% means that the two markers are unlinked. This can be caused by the two markers being distant from each other on the same chromosome, but more often because they are on different chromosomes altogether. A θ of 0% means that the two markers are tightly linked. The genetic distance unit is centiMorgan (cM). If the recombination fraction of markers A and B is 10%, they are said to be 10 cM apart from each other on the same chromosome or LG.

The statistic used to estimate the likelihood that two markers are linked is the logarithm of odds (LOD) score. Higher LOD score means better likelihood of real linkage between two markers and a more accurate estimate of the genetic distance between them. The two factors affecting the LOD score are the number of informative meioses or informative individuals (N) in the mapping panel and the observed recombination distance (θ). Small θ and large N contribute to a higher LOD score. As a rule of thumb the arbitrary linkage threshold of LOD = 3 (a 1 in 1,000 chance of an error) is widely used for constructing linkage maps, but more careful attention should be taken when small panels ($N < 50$) and/or large recombination distances ($\theta > 0.20$) are being considered. For a much more detailed discussion and very informative examples and illustrations of these aspects of genetic mapping, the readers are referred to the chapter written by Danzmann and Gharbi on linkage mapping in the book *Aquaculture Genomics Technologies* (Chapter 10; Liu 2007).

Cross-over interference is the term used for describing the tendency of neighboring recombination events along the chromosome to decrease the frequency of each other (i.e., they cannot be treated mathematically as independent events). The degree of interference is affected by chromosome structure and size as well as genetic regulatory factors. Several mapping functions were developed to correct for interference when multiple cross-over events occur between two markers. The most widely used are called the Haldane and the Kosambi functions (Haldane 1919; Kosambi 1944). Empirical data suggest that the degree of interference is higher near the centeromere of the chromosome. LGs with a high incidence of multiple cross-over events tend to correspond to large metacentric chromosomes, and lower incidence tends to be associated with short and acrocentric chromosomes. However, large differences in the rate of interference occur even between chromosomes of similar size and structure. Gender is another factor that can have a large impact on cross-over interference. The informative markers from the site can be arranged in a male map and from the dam in a female map. The length of each LG and the overall length of the map are greatly affected by interference. Typically in fish the male map is shorter. In salmonids, the length differences between the male and female maps are especially large (see Danzmann et al. 2005 for further discussion).

The typical panels for linkage mapping in aquaculture species are composed of backcross families, F2 intercross or pedigreed outcross families. Another unique resource used in rainbow trout genomics is a panel of doubled-haploid fish produced using gynogenesis (e.g., Guyomard et al. 2006) or androgenesis (e.g., Young et al. 1998; Nichols et al. 2003a). First, a hybrid line is prepared by crossing two genetically different homozygous (clonal) lines produced using mitotic gynogenesis or androgenesis (e.g., Young et al. 1996). The hybrid line is then manipulated further by gynogenesis or androgenesis to produce homozygous doubled haploids, which represent meiotic recombination events between the two parental clonal lines. The major advantage of the doubled-haploid panel is that all the heterozygous markers in the hybrid genome are informative for mapping (including dominant markers).

Numerous computer programs for genetic linkage analysis are available. The most widely used in aquaculture research are MAPMAKER (Lander et al. 1987), CRIMAP (http://linkage.rockefeller.edu/soft/crimap/), JOINMAP (Stam 1993), and LINKMFEX (Danzmann and Gharbi 2001). A short and very informative description of the features of those programs is given in the chapter written by Danzmann and Gharbi on linkage mapping in the book *Aquaculture Genomics Technologies* (Chapter 10; Liu 2007). Another good resource for information on and links to genetic analysis programs is available online from the bioinformatics group of the USDA national research support program 8 (NRSP-8) (http://www.animalgenome.org/bioinfo/).

Moderate- to high-density linkage maps are now available for all five species groups identified as high priority for US aquaculture genomics research (Alcivar-Warren et al. 1997). Most of the earlier maps were based on AFLPs or a mixture of microsatellites and AFLPs, but recently high-density microsatellite-based maps became available for tilapia and trout (Lee et al. 2005; Guyomard et al. 2006). The following is a partial list of published maps for each of the five species groups.

Salmonids: Young et al. (1998), Sakamoto et al. (2000), Nichols et al. (2003a), Moen et al. (2004, 2008), Danzmann et al. (2005), and Guyomard et al. (2006).
Tilapia: Kocher et al. (1998), Agresti et al. (2000), and Lee et al. (2005).
Catfish: Waldbieser et al. (2001) and Liu et al. (2003).
Shrimp: Wilson et al. (2002), Li et al. (2003, 2006), and Perez et al. (2004).
Oyster: Li and Guo (2004) and Hubert and Hedgecock (2004).

Linkage maps were also published for Atlantic halibut (Reith et al. 2007), Ayu (Watanabe et al. 2004b), carp (Sun and Liang 2004), European sea bass (Chistiakov et al. 2005), gilthead sea bream (Bargelloni et al. 2007), Japanese flounder (Coimbra et al. 2003; Castano-Sanche et al. 2007), loach (Morishima et al. 2007), and Yellowtail (Ohara et al. 2005). Marker development and genetic mapping efforts are under way for other economically important species such as Atlantic cod (Delghandi et al. 2007), striped bass (Rexroad et al. 2006), and red drum (Karlsson et al. 2008).

Detection and Mapping of QTL

A QTL is a segment of the chromosome that contributes to the additive genetic variation of the phenotype. It is mapped to a specific segment of an LG by linkage analysis of the phenotype and previously mapped genetic markers. The expectation is

that significant differences in the mean phenotypic values of the trait will be observed between genotypes in a segregating family if a marker or several markers are linked to the QTL.

Current selective breeding in aquaculture is mainly focused on growth and body size (Gjedrem 2000; Gjerde 2005) that are relatively easy to measure and have high heritability. However, in the more advanced segments of aquaculture there is interest in genetic improvement of complex traits such as disease resistance, feed efficiency, stress response, flesh quality, and fillet yield that are expensive and hard to measure and often exhibit low heritability. The promise of QTL detection is that it will eventually lead to fine mapping of markers tightly linked to the DNA sequence variation affecting the trait and to indirect selection using the marker alleles linked to improved performance (marker-assisted selection or MAS). Further analysis and dissection of the molecular data can lead to the identification of the actual gene or genes affecting the trait. This information may then be transferable across populations of the same species and maybe even across species, and will improve our basic understanding of the biology of the trait and the organism.

The main motivation behind intensive marker development efforts and the production of genetic linkage maps for aquaculture species has been to obtain the necessary tools and background information needed for QTL detection. If the genetic map is dense enough, it enables the selection of evenly spaced markers for linking the QTL to specific marker intervals. The efficiency of QTL detection can be improved by thoughtful experimental design and the use of computer programs accounting for possible pleiotropy (multitrait effects of a single locus), epistasis (complex multilocus effects on a trait or traits), and linkage of two or more QTL.

An impressive number of QTL identification studies in aquaculture species have been published, mostly for rainbow trout and tilapia. Some examples in rainbow trout include resistance to the pathogen *Ceratomyxa shasta* (Nichols et al. 2003b), resistance to IHNV (Khoo et al. 2004; Rodriguez et al. 2004; Barroso et al. 2008), resistance to IPNV (Ozaki et al. 2001, 2007), killer cell-like activity (Zimmerman et al. 2004), upper thermal tolerance (Perry et al. 2001, 2005), embryonic developmental rate (Robison et al. 2001; Sundin et al. 2005; Nichols et al. 2007), spawning time (Sakamoto et al. 1999; O'Malley et al. 2003), and smoltification (Nichols et al. 2008). Some tilapia examples include QTL for cold tolerance and growth (Cnaani et al. 2003), growth rate, sex determination, and stress response (Cnaani et al. 2004), sex ratio distorters (Palti et al. 2002; Shirak et al. 2002), sex determination (Lee et al. 2003), and survival, sex determination, and body coloration (Lee et al. 2005).

BAC Libraries and Physical Mapping

Large-insert genomic libraries are essential for genomics research in aquaculture species, particularly in the absence of whole-genome sequence. BAC (Shizuya et al. 1992) is the most stable cloning vector commonly used for constructing large-insert genomic libraries. Physical genome maps are assembled using BAC DNA fingerprinting of all the individual clones in a library and can be aligned with or cross-referenced to genetic linkage maps. Such integrated physical and genetic maps are useful for fine mapping of economically important QTL and for surveying the BACs that harbor the QTL for candidate genes that affect the trait of interest (Grisart et al. 2002).

BAC physical maps are also used to provide minimal-tiling path for whole-genome sequence obtained with the clone-by-clone approach, or as a frame-work scaffold for identifying and filling in gaps in a genome sequence obtained using the whole-genome shotgun (WGS) approach (see the DNA sequencing technologies section). Additional applications of BAC libraries and physical maps include isolation and characterization of genes and their genomic architecture, isolation, and development of microsatellite markers from BACs that harbor genes of interest and from BES and comparative genomics to identify homology to sequenced genomes and also chromosome rearrangements between related species (e.g., among salmonids) using BACs as probes for fluorescent in situ hybridization (FISH; Phillips et al. 2006). Typically, the average insert size in BAC libraries is between 100 and 200 kb, and they contain severalfold coverage of the genome to maximize the number of genome loci that are archived in the library. The coverage is estimated by multiplying the number of BAC clones by the average insert size. For example, if the organism genome size is 3×10^9 bp and the average insert size in the library is 150 kb, then to obtain ten times (or 10×) genome coverage the library has to be composed of 200,000 clones.

BAC Library Construction

High-molecular-weight genomic DNA is partially digested with a restriction enzyme suitable for ligation into the BAC vector (typically HindIII, EcoRI, or BamHI). An alternative protocol, available at the listed web site, uses random shearing of large fragments of the genomic DNA to improve representation of the entire genome (http://www.lucigen.com/catalog/images/pdfs/newsletters/Random_ShearBAC.pdf). The DNA fragments are then separated by pulse field gel electrophoresis and the fragments of the desired size (typically 100–250 kb) are excised from the gel, ligated into the BAC vector, and transformed into the designated *E. coli* cell line (Osoegawa et al. 1998; Wu et al. 2004). The individual clones are arrayed into 96- or 384-well plates, and screening tools are prepared to enable the detection of BACs that harbor genes or markers of interest. Nylon filters stamped with BAC clones in duplicates are used for probe hybridization, the most common screening tool. Pools of clones from plates, rows, and columns can also be prepared for PCR screening of the library. PCR pools require elaborate preparation, but they eliminate the need for radioisotope-labeled probes. The source of the genomic DNA is a very critical parameter as well. The library is much more useful for assembling a physical map if the genetic variation of the DNA donor is very low. This is especially important in salmonids that have a recent history of tetraploidization; therefore, lines of doubled haploids have been established that can be used as donors (Young et al. 1996; Palti et al. 2004). It is also important that the donor is a viable representative of the biology of the species and a sustainable source of DNA that can be used for other genomics resources such as EST libraries and whole-genome sequencing.

Characterization

The first step prior to using BAC libraries for costly downstream applications is the characterization of the library to assess its true genome coverage and utility for physical

mapping. The average insert size is estimated using pulse field gel electrophoresis of a subset of clones sampled at random and digested with NotI (rare cutting site of eight nucleotides). Typically, at least 100 BACs are sampled and the range of size distribution is evaluated as well. The genomic representation of the library is evaluated by membrane hybridization or PCR superpools screening for the presence of a panel of genes and markers, preferably from loci that represent all the chromosome/LGs of the organism. The number of "positive" BACs per locus is an indicator of the library's genome representation. The expectation is that a 4× coverage library will have on average of four positive BACs per locus, a 10× library will have ten positive BACs per locus, and so on. However, it is also expected that a small fraction of the genome segments will not be represented in a BAC library due to very low or high restriction sites in the sequence of some genome segments or because of cloning instability that certain segments may cause in the *E. coli* host cells. For species with a duplicated genome, such as salmonids, it is important to follow the library screening by DNA fingerprinting of the positive BACs to identify duplicated loci. Typically, the BACs positive for a single-copy locus will assemble into one contig, but if the locus is duplicated they will be assembled into two DNA fingerprinting contigs (see Palti et al. 2004). The DNA fingerprinting also enables estimates of the number of identical BACs in the library. Other parameters that should be measured are the number of empty wells (clones that did not grow or have very poor growth) in the library and the percent of clones that do not contain an insert. Construction and characterization of BAC libraries of aquacultured finfish were published for Atlantic salmon (Thorsen et al. 2005), catfish (Quiniou et al. 2003; Wang et al. 2007), rainbow trout (Palti et al. 2004), oyster (Cunningham et al. 2006), and tilapia (Katagiri et al. 2001).

Physical Maps and BAC DNA Fingerprinting Methods

A BAC DNA fingerprinting map is an ordered restriction fragment map of overlapping BACs that are arranged into continuous groups or contigs. During the construction and characterization of the BAC library, the goal is to ensure that enough redundancy exists to achieve optimal genome representation and coverage. The physical map is assembled to identify the smallest number of BACs needed to represent the entire genome (also known as "the minimal tiling path"). Coulson et al. (1986) assembled the first published eukaryote whole-genome restriction enzyme map using a two-enzyme digestion of a *C. elegans* cosmid library. The relatively small fragments were separated on polyacrylamide gels. During the same year, Olson et al. (1986) mapped the genome of the yeast (*Saccharomyces cerevisiae*) using a single-enzyme digestion and then separated the large fragments on agarose gel. The agarose method refined by Marra et al. (1997) and coupled with the development of the FingerPrinted Contigs (FPC) software by Soderlund et al. (1997) enabled the construction and assembly of the complex genomes of higher eukaryotes (Marra et al. 1999; International Human Genome Sequencing Consortium 2001). Although the agarose gel method has been very successful and is still used to construct physical maps, more high-throughput automated methods that employ several restriction enzymes and take advantage of improvements in fluorescent labeling and capillary electrophoresis have been developed in recent years (Ding et al. 2001; Luo et al. 2003). As a necessity, these methods are coupled with improvements to the FPC software package (Nelson et al. 2005).

These methods are collectively called high-information content fingerprinting, and another advantage they have over the "traditional" agarose method is that they can effectively utilize BAC libraries with an average insert size smaller than 150 kb for constructing physical maps. Published BAC fingerprinting maps of aquacultured species are available for Atlantic salmon (Ng et al. 2005), catfish (Quiniou et al. 2007), and tilapia (Katagiri et al. 2005).

Genetic Markers from BACs

Integration and alignment of the physical map with a high-density genetic map of the same species and/or reference genome sequence from a closely related species is essential for the ordering of the contigs relative to each other and for "positional cloning" of QTL and candidate genes. Microsatellite markers from the genetic map can be anchored to the physical map by PCR screening of the BAC library superpools, if available. Another option is to screen the high-density filters with type I (EST) markers or with probes made from the flanking sequences of microsatellite markers. For better throughput and improved efficiency, the probes can be arrayed in a matrix and pooled in a scheme that enables identification of the individual positive BACs (Romanov et al. 2003). However, this approach was tried in salmonids and the results proved very difficult for analysis, as a significant number of the microsatellite flanking regions still contained repetitive sequences (Davidson et al. and Palti et al., unpublished data). An alternative approach is to identify markers from BACs of interest and then map them back onto the genetic linkage map. Some PCR methods were developed for screening of subclones from a BAC shotgun library for microsatellites (Waldbieser et al. 2003; Rodriguez et al. 2006), but they are fairly low throughput and mainly designed to map candidate genes onto genetic linkage maps (e.g., Coulibaly et al. 2006a; Palti et al. 2006). A more robust approach for identifying microsatellites and other markers in BACs is through BES.

BAC End Sequencing

The ends of the BAC insert can be directly sequenced using the universal T7 and SP6 primers that flank the BAC vector insertion site. Although the end sequences are typically shorter than 700 bp, they provide useful information. BES projects involve sequencing a large portion of the BAC library and creating a valuable database. The best published example to date in aquaculture is from catfish, where BES were shown to be useful for mapping ESTs onto the physical map, anchoring BACs to the sequenced genomes of model fish species, and identifying microsatellites that can be used to anchor BAC contigs to genetic LGs for integration of genetic and physical maps (Xu et al. 2006; Somridhivej et al. 2008). BES are also very useful for validation of BAC order in the DNA fingerprinting contigs and for fine-tuning of the minimal tiling path. To accomplish that, sequence tag sites (STSs) are generated by designing unique PCR primers from end sequences of several representative BACs from the fingerprinting contig. The BACs from the contigs of interest are then screened using the STSs to validate the fingerprinting contigs and fine-tune the minimal tiling path (e.g., Shay et al. 2001).

Chromosome Walking and Positional Cloning

BAC physical maps integrated with genetic maps can readily be used for identifying contigs that harbor the gene(s) that affect the trait of interest. Then this information can be used to ultimately pinpoint the DNA sequence variation affecting the trait. These processes are called chromosome walking and positional cloning. Chromosome walking is the process of moving along the chromosome toward the gene or sequence variation affecting the trait of interest by developing new markers and remapping using the new markers with the expectation that they will have a higher LOD score than the markers from the last step. Positional cloning is the process of fine mapping a QTL to a region of the chromosome that is small enough to be cloned and sequenced. Sequence information of the specific DNA sequence containing variation enables researchers to pinpoint the trait of interest in the mapping population. Pinpointing the actual sequence variation is important to ensure that the desired alleles are selected in the breeding population and to enable transfer of the QTL information to other populations from the same species, and sometimes even across closely related species. The identification of genes that affect economically important traits such as feed efficiency and disease resistance also enables research involving functional studies for better understanding of the underlying physiology of the organism. The best example in fish genomics for the use of an integrated physical and genetic map for identifying a minimal BAC contig region and sequencing of the region to identify the gene of interest comes from the identification of the sex determination gene in medaka (Matsuda et al. 2002).

ESTs from cDNA Libraries

ESTs are the sequences of complementary DNA (cDNA) clones from cDNA libraries (Adams et al. 1991). The main goal of EST projects is to generate a large database that represents the trancriptome of the organism. ESTs databases are a valuable resource of type I markers for linkage maps, for integration of the physical and genetic linkage maps, and for comparative genome mapping. ESTs and the cDNA clones from which they were derived are also the building blocks of microarrays for functional genomics research as described elsewhere in this book (Chapter 4).

Construction of cDNA Libraries

cDNA libraries represent the expressed mRNA from adult tissues or embryonic developmental stages. The physiological and immunological conditions of the individual animal sampled have significant impact on the RNA content as well. For example, fish that are infected with a virus are likely to undergo inflammatory responses and have high expression levels of cytokines, while the expression of other genes that are involved in other metabolic pathways is likely to be suppressed. Therefore, the source of the RNA is the first thing to consider when ESTs databases are generated for genomics research. Several protocols and commercial kits are available for making cDNA libraries in addition to companies that offer customized production of cDNA

libraries. The common element in cDNA synthesis is the use of reverse transcriptase to retranscribe single-stranded DNA from the mRNA molecules in the first-strand reaction. Oligo(dT) is used as a primer for the reverse transcriptase as it complements the polyA tail of mRNA molecules. Then double-stranded DNA is made by using random primers or 5′ linkers, and the cDNA molecules can be cloned into vectors and transformed into an *E. coli* host of choice. Prior to cloning, however, the libraries are typically normalized by denaturing and reassociation at high temperature and degradation of the double-stranded DNA with an enzyme to reduce the representation of abundantly expressed house-keeping genes. The cloned cDNA molecules are sequenced using sequencing primers that flank the insertion site of the cloning vector. Although the sequence obtained from each clone (typically 600–700 bp) does not represent the full-length cDNA of most genes, it is sufficient for the downstream analyses described below. If a specific EST is of interest for further analyses, additional sequence of the specific cDNA can be obtained from the clone of origin by primer walking. In mature EST sequencing projects, previously sequenced libraries or the sequenced clones from a large mixed library can be used in subtractive hybridization to remove cDNA molecules with the same sequences and increase the representation of novel ESTs for further sequencing.

Gene Discovery

EST databases provide an excellent resource for gene discovery through sequence homology searches (BLAST; Altschul et al. 1990) using gene sequences that have an important role in metabolic and immune pathways in model organisms. This enables further genomic and functional characterization of genes that are likely to have important roles in growth and development or immune response and are therefore candidate genes for affecting aquaculture production traits (e.g., Gahr et al. 2004; Rodriguez et al. 2005; Coulibaly et al. 2006b).

Expression Profiles

Sequencing of nonnormalized cDNA libraries is useful for identifying and quantifying the expression of genes in specific tissues or developmental stages. To date, the examples from fish and aquaculture genomics are mainly focused on the discovery and identification component, while the quantification component relied heavily on using cDNA microarrays. However, with the development and application of new and very robust sequencing technologies (see the DNA sequencing technologies section) the quantification component appears more feasible and affordable. Published examples of the use of ESTs to identify the genes expressed in specific tissues come from zebrafish (Song et al. 2004) and for aquaculture species, predominantly from catfish (Karsi et al. 1998, 2002; Cao et al. 2001; Kocabas et al. 2002). Examples for the use of ESTs to identify genes expressed in early embryonic development come from zebrafish (Lo et al. 2003), medaka (Kimura et al. 2004), and recently rainbow trout (Qiu et al. 2008; Ramachandra et al. 2008).

Type I Markers

EST databases are great resources for developing type I markers for comparative genome mapping, but also simply as a source for rapid development of new genetic markers. Microsatellites found in the ESTs (typically 3′ UTR) are obviously an excellent source for genetic markers development (Serapion et al. 2004; Coulibaly et al. 2005; Rexroad et al. 2005), and SNPs can be detected from clustering and alignment of the sequences in the database (He et al. 2003; Hayes et al. 2007).

Published EST Databases

EST databases are now available for all five species groups identified as high priority for US aquaculture genomics research (Alcivar-Warren et al. 1997). A snapshot of the number of sequences and clusters in the public databases is given in Table 5.1. The following is a list of publications documenting the development and characterization of EST databases for the major aquaculture species:

Atlantic salmon: Rise et al. (2004) and Adzhubei et al. (2007).
Rainbow trout: Rexroad et al. (2003) and Govoroun et al. (2006).
Catfish: Li et al. (2007).
Tilapia (cichlids): Watanabe et al. (2004a) and Salzburger et al. (2008).
Shrimp: Tassanakajon et al. (2006) and Leu et al. (2007).
Oyster: Quilang et al. (2007).

Table 5.1. ESTs from aquaculture species (or closely related species) in public databases as of July 18, 2008.

	NCBI (http://www.ncbi.nlm.nih.gov/)		TIGR/CBFGL Gene index (http://compbio.dfci.harvard.edu/tgi/)	
Species	ESTs	UniGene[1]	ESTs	TC[2]
Atlantic salmon	433,337	31,957	432,243	49,630
Rainbow trout	260,887	25,025	258,973	40,320
Cannel catfish	44,767	N/A	44,328	5,342
Cichlids				
Haplochromis burtoni	10,312	N/A	N/A	N/A
H. chilotes	N/A	N/A	20,633	2,291
H. red tail	N/A	N/A	13,731	1,942
Oyster				
Pacific oyster	29,018	N/A	N/A	N/A
Eastern oyster	14,560	N/A	N/A	N/A
Shrimp				
Penaeus monodon	8,073	N/A	N/A	N/A
P. chinesis	10,446	N/A	N/A	N/A

[1]UniGne sequence clusters.
[2]Tentative consensus (TC) sequences.

Comparative Genome Mapping

Comparative genome mapping can be used to study the evolution of teleosts and vertebrates through similarity and differences between species in their genomic organization and gene content, but it can also be used as an effective tool in positional cloning of QTL. With the current cost of DNA sequencing technologies and the complexity and size of the genome of most aquacultured species, it is unlikely that a complete whole-genome sequence will be available for all species. However, the genome sequence of several model fish species (fugu, tetraodon, zebrafish, medaka, and soon three-spine stickleback and tilapia) can be used to identify chromosome segments of several million base pairs that are similar between the aquacultured organism of interest and the model species. Once a QTL-containing region is narrowed to several million base pairs and aligned with BAC contigs, segments of synteny that are similar in gene content and order can be identified on the genome sequence of one of the model species and used to refine the genomic map of the QTL and to identify candidate genes for positional cloning.

Genetic Linkage Maps

Comparative genome mapping can be achieved using genetic maps if the species compared are close enough to share genetic markers (mainly microsatellites). This can be done by constructing a separate map to each species using the same markers and comparing the location and order of the markers with respect to each other (e.g., in salmonids; Danzmann et al. 2005) or by using interspecific hybrids (see Liu et al. 2003 for catfish and Lee et al. 2005 for tilapia).

Expressed Sequence Tags

Sequence homology between ESTs and the genome sequence of model species make them an ideal marker for comparative genome mapping. If the ESTs contain microsatellites (Serapion et al. 2004; Rexroad et al. 2005) or SNPs can be identified in the mapping population (Moen et al. 2008), they can be mapped onto the genetic map and used to anchor the genetic LGs onto chromosome segments of the sequenced genomes.

BACs and Physical Maps

In closely related species, BAC and physical maps can be anchored to the sequenced genome of model organisms by screening the BAC library filters or PCR super-pools using ESTs or other sequence markers from the model organism. A good example for that is the use of chicken sequences to generate "overgo oligos" for screening the turkey and zebra finch BAC libraries (Romanov and Dodgson 2006). BES with sequence homology to specific loci on the genome sequence of the model species can also be used to anchor individual BACs or a contig from the physical map onto the genome sequence.

Fine Mapping and Positional Cloning

Lee and Kocher (Liu 2007) describe how they used the synteny they identified between the sex-determining region on LG1 of the Nile tilapia genetic map and a segment of Chromosome 5 of Tetraodon to refine the map of the sex-determining region toward positional cloning of a sex-determining gene in Nile tilapia. This is an excellent example on how comparative mapping can be used in conjunction with the genetic map, the BAC physical map, and the EST database. The locus was initially mapped to an interval of 11 cM on LG1 (Lee et al. 2003). One of the markers flanking the interval was used for screening of the BAC library, and positive BACs from a single contig of the physical map were identified. Partial sequencing of a four-BAC minimal tiling path of the contig was conducted by shotgun sequencing of 100–200 subclones from each BAC. The shotgun tilapia sequences consistently matched annotated genes from a segment of Tetraodon chromosome 5, and sequence similarities were also identified between cichlid ESTs and some of the annotated genes from the Tetraodon chromosome segment. The ESTs were used to identify additional BAC contigs that align with the chromosome segment and to develop new genetic markers for fine genetic mapping of the sex-determining locus. The additional BAC contigs were sequenced, and gaps between the contigs closed by chromosome walking.

DNA Sequencing Technologies

The ultimate map of every genome is the complete DNA sequence of the genome. Obviously, the feasibility of sequencing the large genomes of aquaculture species has a lot (if not everything) to do with the continuous improvement of DNA sequencing technologies both in terms of throughput and capacity and in terms of cost and affordability. The dramatic improvements in DNA sequencing technology coupled with the continuous improvements of computer processing power, storage capacity, and networking technology have been the driving force behind the genomics revolution in medical and life sciences research over the past three decades. Here are brief descriptions of the concepts behind the Sanger dideoxy method, the current gold standard of DNA sequencing technologies, and the emerging pyrosequencing methods.

Sanger Sequencing

The Sanger sequencing method uses ddNTPs to terminate the elongation of the DNA strand by the enzyme DNA polymerase (Sanger et al. 1977). The ddNTPs are analogs of dNTPs that instead of a hydroxyl group (OH) on the ribose 3′ carbon have hydrogen (H). They are recognized by DNA polymerase and incorporated into the elongated DNA strand, but because they lack OH at the 3′ end their incorporation terminates the elongation reaction. The Sanger method took advantage of the DNA polymerase properties of using a primer and a single-stranded DNA as a template to replicate the DNA. The innovation of the method was to use a certain proportion of ddNTPs and dNTPs in the reaction mix that enabled elongation and termination at every base position along the DNA molecule when sufficient amount of template was used.

Prior to the discovery of the thermostable *Taq* DNA polymerase and the consequent widespread use of PCR, the DNA sequencing reaction required cloning of the DNA template in vectors that produced single-stranded DNA. With PCR, the double-stranded DNA template is denatured at high temperature and the primer is annealed and elongated at lower temperature. The main difference between the current Sanger sequencing reaction and PCR is that in sequencing a single primer is used and in addition to dNTPs the reaction mixed contains ddNTPs in a specific proportion. In its original form, the sequencing reaction of each DNA sample was done in four tubes. In the "A" tube, the reaction mix had ddATP in addition to the four dNTPs, in the "C" tube it has ddCTP, and so on. The primers were typically labeled by radioisotopes or fluorescent dyes and the content of each tube was run in a separate lane on a polyacrylamide gel, creating a classic ladder formation on the gel. The DNA sequence was deduced from the position of the bands relative to the other bands in all four lanes. This has also been dramatically improved and automated by the Applied Biosystems (ABI) sequencing platform that takes advantage of the four-color fluorescent labeling system. In the ABI single-reaction single-lane sequencing, the ddNTPs are labeled, each type by a different color; the fragments of sequencing reaction product are resolved by gel or polymer electrophoresis and the color of each of the separated sequencing fragments is revealed by a laser detector and automatically analyzed by the sequence analysis software on the computer connected to the Genetic Analyzer instrument.

Although Sanger sequencing has been the gold standard of DNA sequencing and has been used to sequence all the different genomes from bacteria to human, it suffers some major limitations. The need to use labeled ddNTPs and electrophoresis makes it prohibitive in terms of throughput and cost. Therefore, tremendous effort and attention were put in recent years into developing new DNA sequencing methods and platforms that will address the research needs required for resequencing many individual genomes in human and other model organisms, and for affordable methods to sequence the genomes of the many nonmodel organisms such as most of the aquaculture species.

Pyrosequencing

The concept behind pyrosequencing is sequencing by synthesis. It takes advantage of the release of pyrophosphate (PP_i) during DNA synthesis that in turn can be converted into ATP in a chain of enzymatic reactions. In the presence of a substrate, the enzymatic reactions generate light emission that can be recorded and quantified. The DNA synthesis is initiated by the hybridization of the sequencing primer to the DNA template in a reaction mix containing DNA polymerase and several other enzymes. The four nucleotides are injected into the enzymatic reaction mix one at a time and the light emission from the incorporation of the correct base is detected. After the detection of light, the enzyme apyrase is injected to clear the system of nucleotides and ATP by degradation and allow for the next cycle involving reinjection of nucleotides and sequencing of the next base position (Ronaghi et al. 1998; Ronaghi 2001). An inherited problem of the process is the sequencing of mononucleotide repeats. The light signal detected is proportional to a certain extent to the number of mononucleotides incorporated in a row, but with the current technology it is not

possible to sequence mononucleotide repeats that are longer than eight bases. At present, the two major limitations of the pyrosequencing technology that prevents it from being used for whole-genome sequencing are the relatively short reads (200–250 bp) and its inability to sequence through long mononucleotide repeats. A major advantage for SNP discovery is the single copy or haploid nature of the pyrosequencing process. This means that in individuals heterozygous to an SNP, each allele will be sequenced separately that makes it much easier to streamline and apply computerized scripts for the SNP detection process.

The 454 Life Science DNA Sequencing

The 454 DNA sequencing platform is based on massive parallel pyrosequencing reactions in picoliter-size vessels. It is automated for greater throughput than the "traditional" ABI platform of Sanger sequencing. Recently in a proof of concept, Wheeler et al. (2008) used the 454 platform to sequence the diploid genome of a single human individual at 7.4-fold redundancy. The sequence of approximately 24.5 billion base pairs was accomplished in only 2 months and at approximately one-hundredth of the cost of the most advanced Sanger sequencing platform. The researchers were able to align the genome sequence to the reference human genome sequence and identify millions of SNPs. Some information on the details of the technology is available from the company's web site at http://www.454.com/index.asp and from a key Nature publication (Margulies et al. 2005). A step-by-step manual of the Genome Sequencer FLX platform with good illustrations can be obtained by contacting the company. Here is a very brief description of the system: the preparation of the single-stranded template DNA (sstDNA) for sequencing is done in seven steps. The first step involves shearing and size selection of the 300–800 bp fractions (or any fraction within that range) from agarose gel. In the second step, the genomic fragments are "polished" for blunt-end ligation. In the third step, two types of adaptors ("A" and "B") are ligated to the ends of the double-stranded DNA fragments. The adaptors' ends are dephosphorylated to prevent adaptor–adaptor ligations. The 5′ ends that provide the unique amplification and sequencing priming sites also have a 5′ overhang, while the 3′ ends are blunt to ensure ligation in the correct orientation. The end of the 5′ strand of adaptor B is attached to biotin. Three types of fragments are formed in the adaptor's ligation step. The first has adaptor A on each side, the second has adaptor A on one side and B on the other side, and the third has adaptor B on each side. In the fourth step, the ligation mixture is immobilized onto magnetic streptavidin-coated beads through the biotin tags on the B adaptors. The fragments flanked by two A adaptors are then removed in a wash procedure. In the fifth step, the gaps or nicks in the 3′ junctions of the adaptors with genomic DNA fragments are repaired using strand-displacing DNA polymerase. The nicks are present because the original adaptors were dephosphorylated. In the sixth step, the sstDNA library is isolated by melting of the single strands flanked by adaptor A on the 5′ end and adaptor B on the 3′ end, as they are not bound to the streptavidin-coated magnetic beads. Fragments flanked by two B adaptors are bound to the beads at both ends and will not release either strand. The magnetic beads are then removed from the mixture. The sstDNA library is then immobilized onto DNA capture beads. The capture process involves hybridization of the B adaptors on the 3′ end of the fragments to complementary oligonucleotides that are covalently bound

to the DNA capture beads. The library of sstDNA fragments is carefully quantified to ensure that only one single-stranded fragment is bound to each bead. The beads are emulsified with PCR reagents in "water-in-oil microreactors" to amplify a clonal single-stranded library from each single-stranded genomic fragment. Following bead recovery, filtration, and enrichment, each bead containing a single-amplified fragment is placed into an individual well of the PicoTiterPlate device, and the incubation mix (containing DNA polymerase and a sequencing primer) is added. After incubation, the rest of the enzymes required for pyrosequencing are added and the parallel synthesis and sequencing of 400,000 DNA molecules occurs in one PicoTiterPlate in one GS-FLX 424 sequencing instrument.

For aquaculture genomics, this platform provides an attractive alternative for applications such as SNP discovery by deep sequencing of reduced representation libraries, whole BACs or BAC contigs minimal tiling path sequencing, and cDNA sequencing to produce expression profiles, as described in the previous segments of this chapter. The platform is still not sufficient for an initial WGS sequencing of a reference genome because of the currently unresolved shortcomings of pyrosequencing, namely, short reads and inability to sequence through long mononucleotide repeats. In addition, it is important to keep in mind that the cost of the instrument and reagents for routine usage are beyond the reach of a typical aquaculture research laboratory. Therefore, collaboration or outsourcing to a core facility will be necessary, at least in the near future.

Solexa DNA Sequencing

Another fast-emerging ultrahigh-throughput DNA sequencing platform comes from Solexa (www.solexa.com or www.illumina.com). This platform is even newer than the 454 technology and has an even higher dependency on a reference genome for resequencing and alignment. It is based on the concept of parallel sequencing as well. The sequence read length is only 36 bp, but millions of sequences are generated per run of the instrument. According to the company's web site, more than a billion of base pairs are sequenced from a single run.

Strategies for Whole-Genome Sequencing

Two strategies for whole-genome sequencing emerged from the human genome project. The first is the hierarchical clone-by-clone approach and the second is the WGS sequencing. The two methods are described with very good illustrations in a review by Eric Green (2001). Although originally the two approaches were in competition with each other, genomics researchers quickly realized that they complement each other so well that the large genome centers currently employ a hybrid approach. The strategies are based on the current ABI platform for Sanger sequencing, but once the 454 and the other alternative technologies catch up and reach the threshold of more than 500 good-quality bases per read the same concepts will apply.

In the clone-by-clone approach, a minimal tiling path is identified from the BAC fingerprinting physical map and the BACs that are part of that path are sequenced by shotgun sequencing. The term *shotgun sequencing* refers to the random shearing of the BAC and subcloning of the smaller fragments (typically 4 kb) into plasmids to provide

5–10 times coverage of the BAC insert. The smaller 4-kb inserts are sequenced using primers that flank them from each end of the cloning vector and the overlapping reads are assembled into one sequence contig using specialized computer programs (e.g., Palti et al. 2007; www.phrap.org). In addition, BES from all the BACs in the library are used to anchor the remaining BACs to the minimal tiling path, to identify gap-closing opportunities and to identify and correct errors in the assembly of the physical map. In the WGS approach, several genomic libraries of varying insert size are prepared and the insert ends are sequenced and assembled into contigs. Typically, an intermediate cosmid library (40-kb insert size) is used in addition to BACs and the 4-kb insert size plasmids. The BAC insert ends serve for anchoring and orientation of large sequence contigs, the cosmid insert ends provide intermediate anchoring and orientation, and the small inserts provide the necessary high genome coverage and redundancy. The WGS approach by itself is faster and less expensive, but its assembly in highly repetitive regions of the genome is more error prone.

Summary and Conclusions

In this chapter, genomics research technologies were broken into seven components and the concepts of each component and the status of the technology in aquaculture research briefly described. A summary of the status of each of those components in the five leading aquaculture species groups in the USA is given in Table 5.2. At present, funds have been secured for sequencing of the genomes of the tilapia by NIH in the USA, and the Atlantic salmon by an international consortium led by Canada and Norway. There is no doubt that the basic genome resources and technological capacity have been developed for some of the major aquaculture species and it is safe to say that the genomics revolution is here.

Table 5.2. Status of tools and reagents for genome research in aquaculture species.

Genome resources	Species that have the resources
Availability of large number of genetic markers (excluding SNPs)	Atlantic salmon, catfish, oyster, rainbow trout, shrimp, striped bass, tilapia
Moderate-density genetic linkage map (with <5 cM resolution)	Atlantic salmon, rainbow trout, tilapia
Published QTL for aquaculture production traits	Atlantic salmon, rainbow trout, tilapia
BAC-based physical maps	Atlantic salmon, catfish, oyster, rainbow trout, tilapia
Merging linkage maps with physical maps to produce integrated maps	Atlantic salmon, tilapia
Large collection of ESTs with a minimal number of 100,000 ESTs	Atlantic salmon, catfish, rainbow trout
Partial or complete genome sequencing	Atlantic salmon, tilapia

Adapted from the NRSP-8 Aquaculture group White Paper.

The large investment in generating tools and reagents for genomics research in aquaculture can easily be justified by the economic and cultural importance of aquaculture around the globe and the use of aquacultured species as model research organisms for ecology, environmental studies, and evolution. However, the large number of aquaculture species makes it unlikely that the genomes of all the species will be sequenced. Rather it will fall upon the scientists to continue to be creative and make the most of opportunities presented by comparative genomics and the new 454 pyrosequencing technology and other robust DNA sequencing technologies that are likely to emerge in the near future. One example for such a creative approach is the use of comparative genome mapping for positional cloning of the sex-determining locus in Nile tilapia by Lee and Kocher.

The ultimate challenge in aquaculture genomics research is linking genotype and phenotype and applying that knowledge to improve production efficiency and sustainability. The impact of genomics on aquaculture production to date has been fairly limited. Examples include the use of markers for parentage assignments in mass spawning or common garden breeding schemes (Johnson et al. 2007; Pierce et al. 2008; and references therein) or for identifying strain of origin (Johnson et al. 2007; Waldbieser and Wolters 2007; Waldbieser and Bosworth 2008). There is no doubt that genomics still has a great potential, but the road to the fulfilled promise is still long and the burden of proof is still on the aquaculture genomics scientists and researchers.

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Chapter 6
Proteomics in Aquaculture

Samuel A.M. Martin

Introduction

Advances in functional genomics are occurring at an ever-increasing rate. This is being driven by several factors including high-throughput sequencing of expressed genes and whole genomes in both model and nonmodel organisms, technological advances in robotics and mass spectrometry, and bioinformatic analysis of generated data. Together this has led to a massive increase in the ability to perform both transcriptomics and proteomics analysis.

The phenotype of an organism is controlled not only by its genes, but also by the environment and the interaction between the genotype and the environment. The result of this complex regulation leads to the ultimate coordinated expression of proteins, now described as the proteome, the study of which is termed proteomics. The proteome is defined as the expressed protein complement of a cell, tissue, or whole organism, with the term *proteomics* being first used in 1994 (Williams and Hochstrasser 1997). The proteome, unlike the genome, varies both temporally and between tissues as the fish grows and adapts its physiology to meet the demands of a new environment. As proteins are the final determinant of phenotype—the proteome that describes the abundance, identity, posttranslational modifications, and potentially the synthesis rates of proteins—an understanding of the regulation proteome is imperative to gain a holistic view of the animal.

This chapter discusses the concept of proteomics and details the technologies related to proteomics that are relevant to all areas of life sciences. The application of these technologies, with an emphasis to aquacultured fish species, is then examined where the important aspects of fish biology including environmental changes, immunological responses, growth and nutrition, and early development have been studied using the proteomics approaches. Details are given of how proteins are identified from fish when only limited genomic information is available. The classic approach to proteomics is studied in most detail, which is separation of proteins first by charge (isoelectric point [pI]) and then by size, termed two-dimensional electrophoresis (2DE). The methodology for this has not changed dramatically over recent years, but the analysis of the gel images that requires considerable computing power has surpassed all expectations. Following analysis of the 2DE gels, selected protein spots are excised from gels and identified by various mass spectrometry approaches. The identification of proteins and accuracy of mass spectrometry is also much improved and linked with nucleic acid sequence databases. Thus, proteomics is proving to be viable approach for research in the aquaculture field.

Proteomics: Relationship between Transcription and Translation

At present, whole-genome sequencing shows that there are about 30,000–60,000 different genes within a eukaryote's genome, with only a percentage of these being expressed in a tissue. Approximately, only 15,000–20,000 genes are expressed in any cell and it takes only a small number of differentially expressed genes to form different tissue types. The scientific dogma that suggests DNA encodes mRNA which encodes protein is now seen as highly oversimplified. mRNA can be alternatively spliced, resulting in more than one final protein for a single gene, but once a protein is translated there are many downstream processes that can modify it further. A common example of this processing is cleavage of a signal sequence from a protein described as "proprotein," as occurs in the plasma protein apolipoprotein. Such signal sequences are required for transport across cell membranes including passage into mitochondria, nucleus, or across the plasma membrane out of the cell. Other posttranslational modifications include phosphorylation of the protein, which relates to the activation or deactivation of a protein, and as such are a highly important regulatory event. Glycosylation is another posttranslational modification where polysaccharides are linked to the protein; these are particularly evident in secreted proteins and those that are processed through the endoplasmic reticulum. Thus, it is possible for a genome of 20,000 potential genes to produce more than 100,000 processed and unprocessed proteins.

Recently, there has been a dramatic increase in genome information available for teleost fish, with the whole-genome sequences made available for zebrafish (Postlethwait et al. 1999), fugu (Aparicio et al. 2002), tetraodon (Jaillon et al. 2004), and medaka (Kasahara et al. 2007). Furthermore, large expressed sequence tag (EST) data sets for nonmodel fish such as Atlantic salmon (Rise et al. 2004; Adzhubei et al. 2007) and rainbow trout (Govoroun et al. 2006) are available, leading to considerable gene discovery in these species in the past few years. This nucleotide sequence information has allowed the identification of individual protein spots isolated from gels to be much more reliable, as seen later in the chapter.

Proteome Technology

Proteome technology is inherently more complex than DNA/RNA technology and for this reason it has not, until recently, been examined in great detail outside the human or model organisms such as Sacromyces and bacteria. The ability to separate proteins with high resolution was developed by O'Farrell (1975), and this approach has led to modern-day 2DE proteomics and its increasing application within the area of fish biology. The key stages of proteome analysis are outlined below (Figure 6.1).

Protein Extraction

Preparation of proteins from the fish is extremely important, particularly to ensure that cellular proteases are inhibited. Protein extraction buffers normally contain urea (8–9 M), 2–4% 3-[(3-cholamidopropyl) dimethylammoniol]-1-propanesulfonate, 25 mM Tris-HCl (pH 7.5), 3 mM EDTA, 50 mM KCl, and 50 mM dithiothreitol; however, there are many variations depending on the tissue type being used. Protease

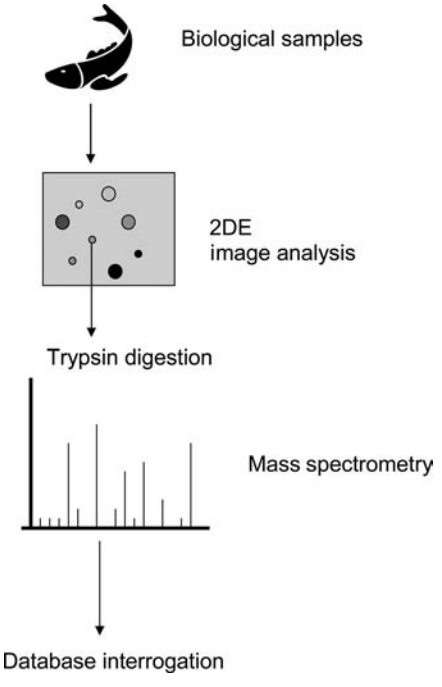

Figure 6.1. Flow diagram of the general approach taken for proteome analysis. Proteins are extracted from the biological samples followed by 2DE and gel image analysis. Protein spots of interest are excised from the gel and subjected to trypsin digestion and separation of peptides on mass spectrometer. Finally, the fragment sizes are used to search protein and nucleic acid databases.

inhibitor cocktails are widely available for use during the extractions. The challenge for these protein extractions is to solubilize as many of the proteins as possible. This is particularly important for the many membrane-bound proteins that are difficult to get into solution. Many hydrophobic proteins are solubilized in nonionic or zwitterionic detergents (Chevallet et al. 1998), and centrifugation is required to remove the remaining insoluble proteins. Further fractionation can be used to enrich or purify cellular organelles (Ho et al. 2006); however, to date this has not been used in fish studies.

Two-Dimensional Electrophoresis

The first dimension in electrophoresis is isoelectric focusing, which separates the proteins on the basis of their charge along a pH gradient. Once the protein reaches a location at which it has no charge, it stops migrating and becomes fixed at this position on the pH gradient. Major advances in recent years have been the use of immobilized pH gradient (IPG) strips that are highly reproducible. Prior to this isoelectric focusing relied on carrier ampholyte pH gradients (Gorg et al. 2004), these strips were technically difficult to make and reproducibility could be low. Now commercially available IPG strips come in many sizes and pI ranges (e.g., pI 3–10 or high-resolution

pI 5–6). For most studies on fish, researchers use either pI 3–10 or pI 4–7. The 2DE is separation by molecular mass using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The combination results in a 2D separation of protein spots, which, following staining, can then be mapped according to the pI and molecular mass. A key technical issue is the reproducibility of gels, which is important for analysis of gel sets not only within a single experiment, but also between laboratories. Between laboratories, gel comparison is still not easily done. It is best to run the gels for an experiment in parallel under identical conditions, but this is not always possible and can depend on numbers of replicates and apparatus available. Equally important is to perform protein extractions at the same time using the same set of buffers. The 2DE gels can separate out hundreds to thousands of individual protein spots with amazingly high resolution when done by experienced hands.

Staining

There are several methods of staining that resolve proteins, the most basic being standard Coomassie blue; although not the most sensitive, it does allow "downstream" processing of spots for subsequent identification. Fluorescent staining methods allow two protein samples stained with dyes that fluoresce at different wavelengths to be loaded onto the gel together. This has the advantage of removing any gel-to-gel artifacts. The most sensitive staining is by silver, although the silver staining often inhibits subsequent analytical steps on the proteins. Once stained, the image is scanned at high resolution and digitized. At this stage, comparison can be made between experimental samples in attempts to identify differentially expressed proteins. A common limitation is the bias toward the abundant proteins, which is a reflection of the dynamic range of proteins being expressed in biological systems.

Image Analysis

The images of stained gels are digitally captured using high-resolution scanners such as the Molecular Dynamics Personal Densitometer (GE Healthcare, Little Chalfont, UK) as 12-bit gray images at a resolution of 50 μm and stored as "*.gel" files, which can then be imported to software packages for gel image analysis. There is continual development of software for 2DE gel analysis, as this is a time-consuming area of proteomics. The first step is to locate the spots on each gel (Figure 6.2); once completed for all the gels in the experiment the gels must be aligned for comparison of the spots. A great deal of effort is required to ensure that the correct spots are aligned, as gel systems, however well run, have subtle differences due to electrophoreses. The Progenesis package (Nonlinear Dynamics, Newcastle, UK) offers excellent gel image analysis with statistical analysis, both within the same program. Whatever package is being used, a gel is chosen to be a "representative gel image"; this is then referred to as a reference or master gel image to which all subsequent gels are matched. The reference gel image may then have additional "virtual" spots added to it relating to protein spots expressed only in other samples.

Matching of spots between gels is started by manual seeding (aligning of obvious spots across the gel for orientation); once a number of spots have been matched,

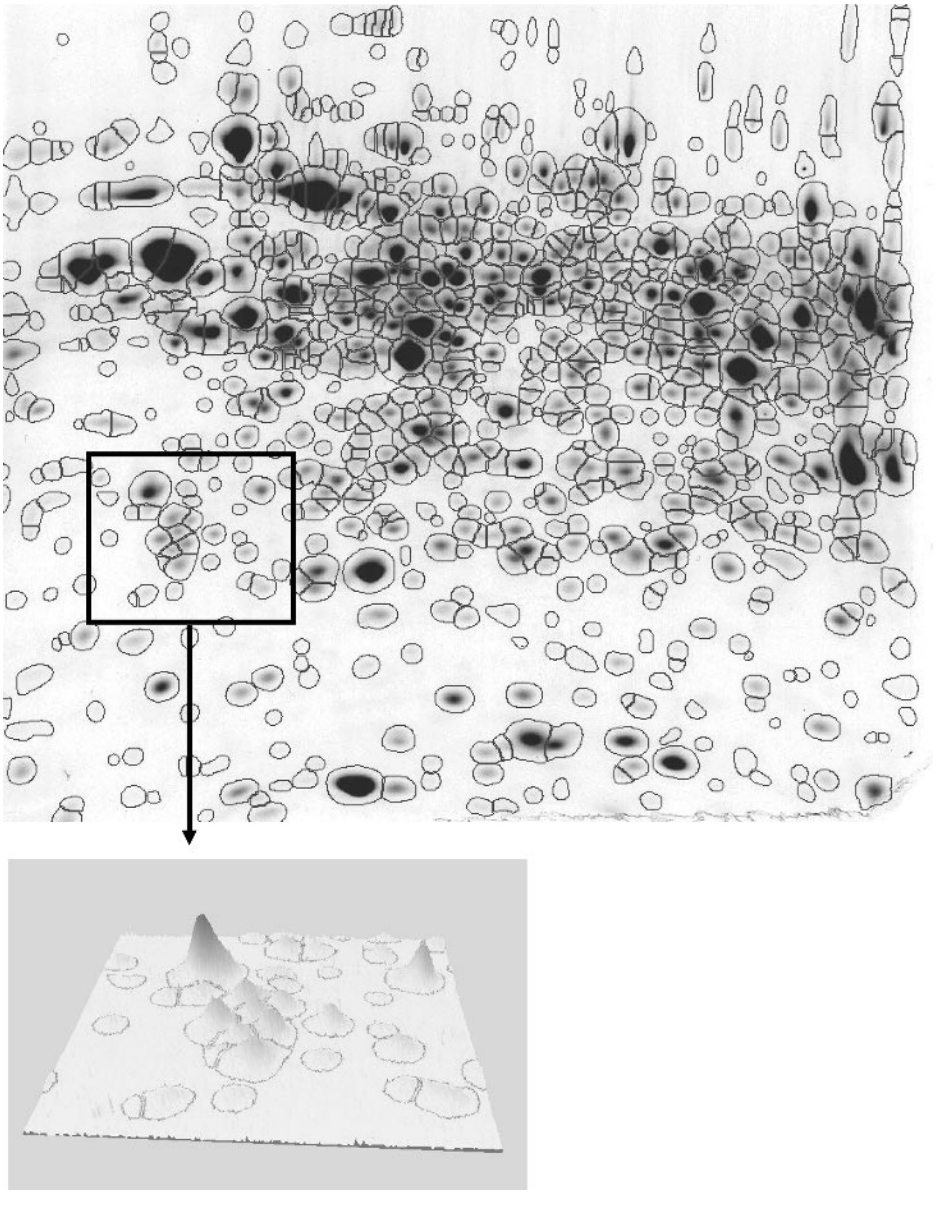

Figure 6.2. 2DE protein gels of rainbow trout liver. Proteins were separated on a pI 4–7 gradient as first dimension followed by size separation on a 10% polyacrylamide gel followed by Coomassie blue staining. Spots have been highlighted by Progenesis software (Nonlinear dynamics). Below is a section of the gel that indicates the volume of the chosen protein spots.

automated matching can be carried out. Gel warping allows the image to be manipulated to allow protein spots to be correctly aligned. However well the software does this matching, it is always necessary to visually confirm that the correct spots have been matched and subsequent manual editing is normally required. Each spot on a 2DE image can then be calibrated according to the molecular weight, charge (pI), and

intensity. The abundance of a protein spot can be determined from the spot volume, which is the area of the spots as determined by the spot search parameters and the intensity of the staining.

Once all gel images are captured and edited, other analysis needs to be performed prior to a gel-to-gel comparison. These include background corrections and normalization between gels, for which there are many different methods for doing this, which are not dealt with here. Statistical analysis of the gels very much depends on the experiment being performed and the numbers of replicates. Simplest methods routinely used are "*t* test" or ANOVA for comparisons between sample groups; however, more complex analysis including principal component analysis can help reveal where the variation between groups is occurring.

The initial analysis of the gels produces data that are sufficient for preliminary statements about expression pattern and abundances; however, to further characterize the proteome, identities need to be given to proteins. Proteins can be sequenced by biochemical methods including Edman degradation. Other proteins can be recognized on a gel following Western blotting and use of specific antibodies; however, the production of antibodies is labor intensive, and as such neither of these procedures is amenable to high-throughput proteome analysis. The use of mass spectrometry, coupled with genomic sequence information, is possibly the area that has most advanced proteome technology in recent years. Both peptide mass fingerprinting (PMF) and de novo sequencing are now routinely performed on fish proteome studies.

Protein Identification by Mass Spectrometry

The current limitations for proteomics in aquacultured fish species are mainly a result of poor sequence coverage of target species and this can be overcome by extended sequencing of the transcriptome. This not only involves EST sequencing but includes production of contigs (creating longer sequences from overlapping EST sequences) from which full open reading frames can be generated, which are required for efficient protein identification. Proteins selected from the 2DE gel for identification are isolated from the gel, unlike the gels used for distinguishing sample differences this is often a gel run specifically for spot cutting and care needs to be taken at this step not to contaminate the sample.

Trypsin Digest Fingerprinting

The identification of proteins from 2DE gels is most commonly achieved by digestion of selected proteins (excised from the gel) with trypsin in order to generate a series of peptides with subsequent separation of these peptides by mass spectrometry. Trypsin digests protein in a highly predictable fashion, resulting in reproducible peptide fragments, normally cleaving a protein following an arginine or lysine residue and the resultant series of fragments is described as a trypsin fingerprint. For separation of these peptides, the most common method of mass spectrometry is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Gevaert and Vandekerckhove 2000). Here peptides are suspended in a matrix of UV-absorbing molecules, ionized by a laser and the resulting ionized molecules are accelerated in

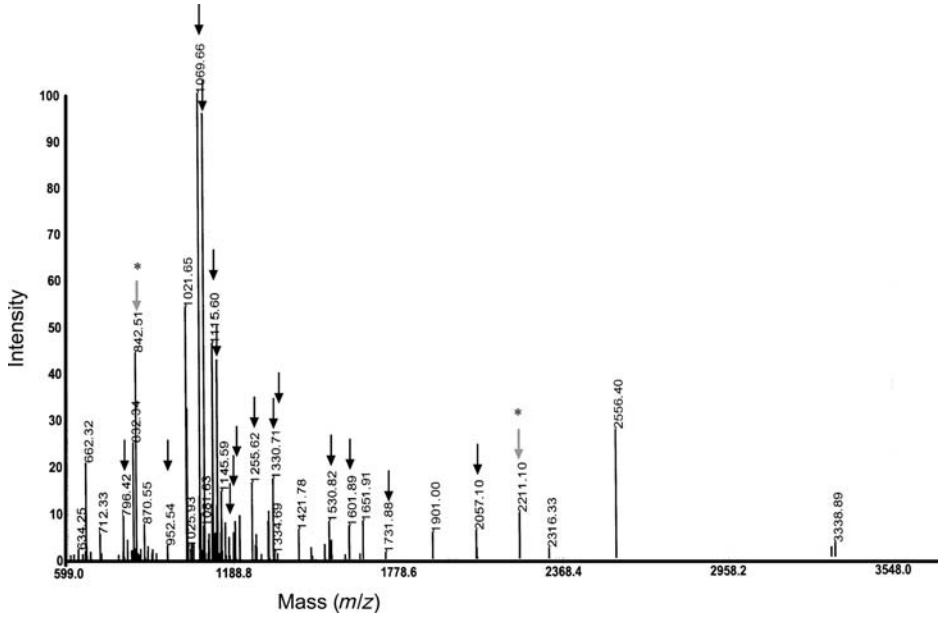

Figure 6.3. Mass spectrometry output for a rainbow trout liver protein found to be differentially expressed. Arrows with ∗ indicate trypsin self-digestion products; other black arrows indicate peptide fragments found to be Apo-AI by database searching.

an electrostatic field where the time of the flight of each fragment is measured, from which the expected mass is calculated. The resulting peptide masses can be visualized as a spectrum in graphic form (Figure 6.3), and the peptide masses used to search various databases which can then give identity to the protein. The occurrence of arginine and lysine residues is random, and not all peptides produce fragments that can be utilized in trypsin digest fingerprinting. Fragments that are too large are not resolved by the mass spectrophotometer, and equally those that are too small are not used either, as they occur too frequently in proteins. Trypsin digest fingerprinting requires a number of peptides to match exactly the target sequence, as a single amino acid change completely alters the mass of a peptide and is therefore not recognized.

The MALDI-TOF MS yields peptide fragments, but higher resolution mass spectrometry can produce amino acid sequence data from the generated peptides. This is achieved when the peptide bonds are broken by high-energy ionization and peptides missing a series of amino acids are separated. As each amino acid has a known mass, the peptide masses produced can be used to infer the amino acid sequence. Liquid chromatography MS/MS (LC MS/MS) is the method of choice for this approach (Figure 6.4). Whichever method of MS is performed the mass tolerance is crucial, which is usually dependent on the error associated with the mass spectrometer. The error allowed is described in parts per million (ppm) and often varies from 50 to 200 ppm in the reported studies on fish. As the error increases, there is more chance of having ambiguous identities.

Once the peptide masses are generated, databases can be examined in an attempt to identify the protein. There are many programs (both freeware and licensed) available for identification of MS data. One of the most straightforward and widely used

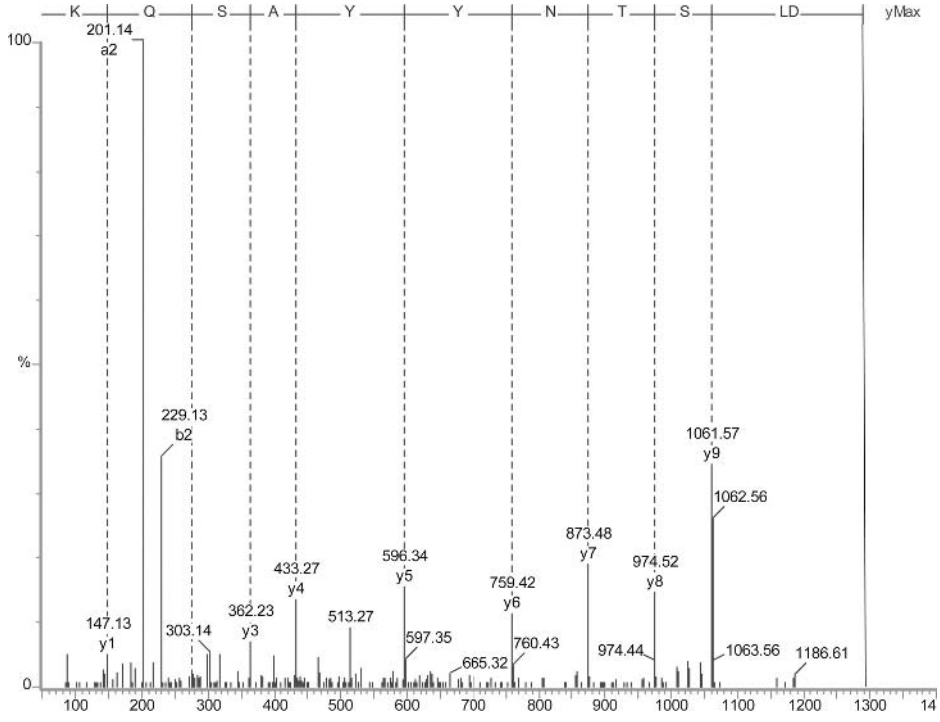

Figure 6.4. Liquid chromatography MS/MS of a protein spot from rainbow trout liver. The sequence obtained was (LD)STNYYASQK. The first two amino acids could not be separated so order of L and D is not known. This sequence was searched against database and found to be a heat shock protein 108.

programs is MASCOT (Perkins et al. 1999) provided by matrix sciences (http://www.matrixscience.com/). This software accepts peptide mass fragments and can search against public protein databases, including Swiss Prot (http://expasy.org/sprot/) and the NCBInr database (http://www.ncbi.nlm.nih.gov/). MASCOT also has the ability to use peptide masses generated by MALDI-TOF MS, peptide sequence coupled with fragment mass, and MS/MS ion searches. Using this approach for protein identification can be problematic for fish as there is a lack of sequence data available and thus relies on peptides showing high levels of conservation across species.

There are still very few protein sequences available for even common aquacultured species such as rainbow trout and carp, which, respectively, have only 2,245 and 1,107 proteins reported (August 2007). However, for many important aquacultured species, the nucleotide sequences for expressed genes are increasing dramatically. This is especially true for the salmonids, Atlantic salmon, and rainbow trout, where there are more than 436,720 and 264,699 nucleotide sequences, respectively (August 2007). Other species including catfish, carp, cod, and sea bass transcriptomes are also being sequenced at a rapid rate. Most of the sequences are ESTs that can be utilized for searching peptide fragments generated by MS. Programs such as MS-Fit (Clauser et al. 1999) will search fragment masses against a nucleotide sequence. To achieve this, the program will perform translations in all six frames of a DNA sequence, which

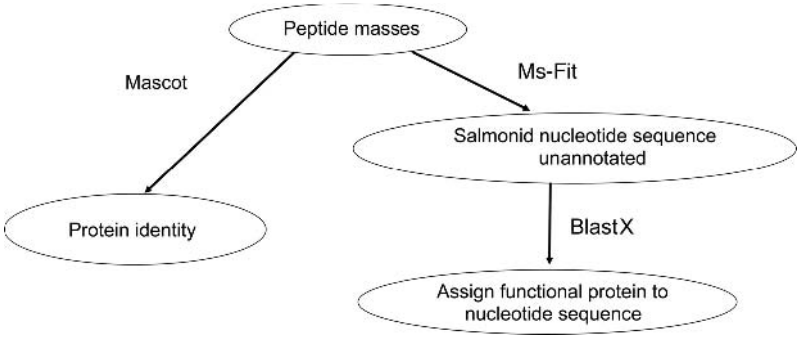

Figure 6.5. Once mass spectrometry data are generated, there are several ways to use this to identify the protein. MASCOT can be used that searches against protein databases; for fish, this results in low percentage of positive hits. An alternative approach is to search against cDNA databases using MS-Fit. Once a positive EST is found, this then requires further searches using BLASTX to identify the protein this gene encodes.

produces hypothetical protein sequences. Each of these sequences is then digested *in silico* with trypsin and the theoretical fragment masses calculated; these are then matched against the test fragment masses. Various parameters can be changed in the search conditions and a molecular weight search (MOWSE) score is produced, which indicates the probability of a positive identification (Pappin et al. 1993). This has been successfully used in rainbow trout (Martin et al. 2003; Vilhelmsson et al. 2004) and Atlantic salmon (Martin et al. 2007b). One problem with using a database generated from ESTs is that many of these sequences do not contain a large open reading frame, as they are single sequence reads from one end of a cDNA clone. This is especially true if the sequencing has been performed from the 3′ untranslated region of the mRNA. Even if the clones were sequenced from the 5′ end, it is likely that the average sequence read is approximately 500 bp, which may include up to 100 bp of 5′ untranslated bases, leaving only 400 nucleotides of expressed sequence that encodes about 130 amino acids.

One step forward has been the cluster analysis and generation of contigs from the EST sequences. There are several groups that have generated contigs for salmonid fish. The consortium for Genomics Research on All Salmonids Program (cGRASP) web site contains data for Atlantic salmon and rainbow trout (http://web.uvic.ca/cbr/grasp/). The Norwegian Salmon Genome Project (http://www.salmongenome.no/cgi-bin/sgp.cgi) has performed clustering and contig assembly for presmolt stages of Atlantic salmon. A third source of ESTs and contigs is the TIGRE database (http://biocomp.dfci.harvard.edu/tgi/tgipage.html), where in addition to Atlantic salmon and rainbow trout there are many other sequence data sets available for fish species. These resources can all be used to help identify the proteins (Figure 6.5).

Environmental Changes—Impact on the Proteome

The environment a fish finds itself in can be subject to dramatic changes requiring adaptation for survival. This is especially true in the aquaculture context where the fish may not always "anticipate" the changes as in naturally occurring populations.

Within aquaculture, environmental alterations can be daily or seasonal and include temperature changes experienced by almost all species of fish, exposure to different salinities as occurs in the rearing of many salmonids, and variable levels of oxygen in the water. Although there are an infinite number of environmental variables that concern aquacultured fish, only these three key factors are examined here as there have been some proteomic studies performed regarding them. Although much of the research is directed at a fundamental understanding of the biology, much can be learned for the culture of fish from the studies.

Osmoregulation

Many species of fish migrate between fresh- and saltwater during their natural life cycle; this is still a fascinating area of research considering how and when fish decide to migrate and also the physiological changes they undergo to adapt to the altered osmotic stresses (Perry 1997). In wild fish, these "decisions" on when to migrate are endogenously made in the brain of the fish in response to day length (among other factors), with the responsive osmoregulatory organs being primed to adapt in a controlled manner in response to hormones. The key osmoregulatory organs are gill, kidney, skin, and intestine. However, in the aquaculture environment, the movement of fish between salinities is determined by the fish farmer, therefore prior knowledge of the protein expression pattern in wild fish will help ensure that correct adaptation can occur.

Salinity changes are known to have dramatic effects on the transcriptional activity in a number of fish species (Gracey et al. 2001; Kultz et al. 2007). Recently, the effects on the proteome have been studied during osmotic changes experienced either by whole animals or on specific cell types. The first efforts to investigate the effects of salinity change on gill epithelium were in the long jawed mudsucker (*Gillichthys mirabilis*) (Kültz and Somero 1996). In this case, the fish were maintained for 2 months at different salinities, either diluted seawater or normal seawater. Proteomics using 2DE was able to distinguish 602 individual proteins, 9 of which were induced following a period in diluted seawater, but no proteins were increased when fish were transferred from diluted to normal seawater. The authors concluded that proteins induced under dilute seawater conditions were important in the function of pavement cells in hyperosmoregulating cells. Unfortunately, during this study no protein identities were assigned to the modulated protein spots.

A more extensive study was performed in the dogfish (*Squalus acanthias*), where four tissues involved in osmoregulation (rectal gland, gill, kidney, and intestine) and two "nonosmoregulatory" tissues (heart and brain) were examined by 2DE (Lee et al. 2006). Although sharks cannot generally survive in freshwater, they often tolerate dilute salinities of 50–70% saltwater. The aim here was to determine whether there was common protein expression between these tissues related to osmoregulatory functions, although no experiments were performed by changing the osmotic conditions of the fish. Following analysis of the dogfish tissues, 270 proteins were excised from gels for MS analysis, and from this 23% (62) of the proteins were identified. Fifty-four proteins were identified using the MS-BLASTP2 program (Shevchenko et al. 2001), whereas 43 proteins were identified using MASCOT, at 50 ppm mass tolerance for both searches. Analysis showed that proteins involved in energy, urea metabolism,

and the Rho-GTPase/cytoskeleton pathway were enriched in osmoregulatory tissues of this shark. An interesting comparison would be between shark and a teleost fish, as the sharks have very different mechanisms of dealing with nitrogenous waste products such as ammonia and urea (Goldstein and Perlman 1995).

One of the key osmotic tissues is the gill, which can be described as a barrier to the hypoosmotic environment. Cultured rainbow trout gill cells have been used to generate intact epithelia on permeable supports (Part and Bergstrfm 1995), allowing detailed analysis of cellular responses. Proteomic analysis has been performed in these primary cultures (Smith et al. 2005), where gill epithelia were maintained as double-seeded; that is, they contained both pavement cells and mitochondrial-rich chloride cells. To help understand freshwater gill function, differential protein expression in these cells was examined following osmotic stress. The integrity of the cell culture was assessed by measuring transepithelial resistance, which confirms that the culture is an impermeable barrier. Control samples (termed *symmetrical cultures*) had culture media on both sides of the cell culture, whereas the experimental cells (termed *asymmetrical culture*) had cell culture media in the basolateral compartment and sterile tap water in the apical compartment. Following 24 hours of culture in this system, proteins were extracted and separated on 2DE gels using standard protocols on a pH 4–7 IPG strip for the first dimension and a 10–15% gradient polyacrylamide gel for the second dimension. Five proteins were found to be significantly increased in the asymmetrical cultures compared with controls. The key protein identified by MASCOT was apolipoprotein AI (Apo-AI), which was increased in abundance by 9.9-fold. The other four proteins were given tentative identifications but were not significant by identity scores. In mammals, Apo-AI is mainly synthesized in the liver and secreted as a major plasma protein. Further work using bovine Apo-AI suggested (Smith et al. 2005) that this protein may play an important role as a determinant of the barrier properties of the trout gill, especially in the modulation of transcellular permeability.

Temperature

Temperature is an environmental parameter that is of central importance to the aquaculture industry, with different species requiring optimal temperatures for maximal growth and health. The effects of temperature change have implications for all biological processes (Nikoskelainen et al. 2004; Podrabsky and Somero 2004). All fish need to be able to adapt to changing temperature, normally on a seasonal basis, but often there can be large daily fluctuations. There is evidence that temperature change involves a two-stage process similar to a stress response (Kultz 2005), where a common set of genes in distantly related organisms shows approximately 300 proteins that are altered. Kultz (2005) suggests that the two stages are the cellular stress response and the cellular homeostasis response. For the cellular stress response a minimal "proteome" has been suggested, which is shared across diverse organisms; hence, care needs to be taken when comparing different experimental protocols. The transcriptome of the eurythermal killifish (*Austrofundulus limnaeus*) (Podrabsky and Somero 2004) revealed groups of genes that were differentially expressed in an acute manner during fluctuating temperature as opposed to acclimation to a constant temperature. In mudsucker (Buckley et al. 2006), key genes related to maintenance of protein homeostasis, cell cycle control, cytoskeletal reorganization, metabolic regulation,

and signal transduction were found to be modulated following temperature change. In experiments on mudsucker (Buckley et al. 2006), a comparison was made between gene expression and protein expression for some key genes found during the transcriptome analysis. The authors performed Western blot analysis on heat-shock proteins (HSP40, HSP70, HSP90), protein disulfide isomerase, and actin. In all cases, there was a parallel induction of both mRNA and protein abundance; however, the magnitude of the response varied quite dramatically, for instance in gill, HSP40 protein increased 8-fold, whereas the mRNA level was induced only 3-fold. Conversely, HSP70 protein in muscle increased 18-fold for mRNA and only 3-fold for protein.

On a carp (*Cyprinus carpio*) microarray, nearly 25% of all genes were altered in expression by temperature (Gracey et al. 2004). In this experiment, fish were cooled over a period of 22 days from an initial temperature of 30°C to either 23, 17, or 10°C. Then multiple tissues were examined for responses indicating large modulations in the transcriptional activity. The same cooling protocol was repeated as mentioned above (Gracey et al. 2004) and a proteomics approach taken (McLean et al. 2007). Here muscle tissue was analyzed and the global protein alterations were examined. Initial analysis was performed only on 1D gels where proteins were separated by size. This was possible as the complexity of muscle is less than many other tissues such as liver and kidney, with a small number of major proteins being present. Identification of proteins by MALDI-TOF MS was performed on trypsin digest products using 250 ppm as the tolerance. The trypsin digest fingerprinting was hampered by the small number of carp protein sequences in the SwissProt database. Additional identification was obtained by de novo sequencing of peptides coupled with fragment masses where LC MS/MS data were used to search the closely related zebrafish nucleic acid database, allowing for much greater identification of proteins. The 2DE analysis was also carried out with further proteins being excised, and the identity of many of these proteins was found to correlate with the proteins excised from the 1D gels. Many of the proteins were found to be enzymes involved in glycolysis, with creatine kinase (CK) being the most abundant protein representing about 25% of all the proteins in the sample. From the 2DE analysis, a number of proteins were found in different locations on the gel at similar molecular masses. The fact that these differed by charge and not size would indicate they were modified by either phosphorylation or glycosylation in a posttranslation modification event. In addition, many proteins shown to be CK were identified between the size range of 20 and 30 kDa with a different pI and molecular mass, suggesting processing by proteases. In other work, similar multiple CK spots have been found in zebrafish muscle (Bosworth et al. 2005). CK is found in tissues that have high-energy requirements and is suggested to be involved in transport and energy buffering (Roman et al. 1997). When an ectothermal animal is cooled, the metabolic rate is likely to decrease and as such it is logical that CK will not be required at such high levels.

The major differences observed in the 2DE gels of carp between warm and cooled samples (42 days acclimation at 10°C compared to control samples maintained at 30°C) were the appearance of additional CK spots of lower molecular mass in the samples from cooled fish. There are three isoforms of CK in carp: M1, M2, and M3, with a predicted mass of 42 kDa. To further investigate the lower-molecular-weight CK fragments, additional MS analysis was performed in order to assign these fragments to particular isoforms. For the M3 isoform, no N-terminal peptides were found, although these had been obtained for the larger M3 protein spots. Other fragments were shown

to be truncated at the C-terminal. On compiling the peptide sequences, a major cleavage site was indicated close to Phe_{278} in the mature protein, although this does not suggest a mechanism for the proteolytic action. A potential mechanism for the degradation of the CK may be by the ubiquitin-proteasome pathway. Western blots of 2DE gels of muscle proteins from either cooled fish or control fish showed that the CK fragments in the cooled fish samples were ubiquitinated and therefore targeted for destruction. Associated with the appearance of the smaller CK fragments, there was a dramatic decrease in the mRNA encoding ubiquitin and other genes encoding proteins related to this pathway. It is suggested that cellular mechanisms of protein degradation for CK are unable to degrade the protein fast enough, and this results in partially degraded protein fragments. These findings are interesting in the context of protein metabolism, which is most likely reduced at lower temperatures, with both synthesis and degradation of proteins being retarded.

Hypoxia

A third major environmental stressor is hypoxia, which again is widely studied in fish and is particularly relevant to the aquaculture industry. Transcriptome studies have been performed on *G. mirabilis* (Gracey et al. 2001) and larval zebrafish (Ton et al. 2003), with both showing large-scale changes in gene expression patterns following periods of hypoxia. Recently, the effects of hypoxia on the proteome have been examined in adult zebrafish muscle (Bosworth et al. 2005). In this case, hypoxic conditions were oxygen tensions of approximately 10% of normal oxygen level, where pO_2 was 140 Torr for control tanks and 16 Torr for the hypoxic tanks. Two different protein extractions were performed either with or without urea, because many of the highly abundant proteins including actins and myosins are solubilized only in homogenization buffer containing 9 M urea. Extracted muscle proteins were separated on IPG strips (pH 5–8) for the first dimension and then by 10% polyacrylamide gels for the second dimension. Following staining, the mean number of spots found on the gels from the nonurea lysis buffer was 323. Forty spots excised from the gels were subjected to standard PMF using MALDI-TOF MS and fragments searched against the NCBInr database using MASCOT at 100 ppm mass tolerance. From this analysis, 29 protein spots were found to have identities to 16 different proteins. The identification of a protein from more than 1 spot on the 2DE gel indicates a large amount of posttranslational modifications as was also noted from the carp during thermal change experiments (McLean et al. 2007). Nineteen of the spots were associated with energy metabolism, including enolase-3 and CK, which were found three and four times at different locations, respectively. The enolase proteins were observed at a similar molecular weight, suggesting either glycosylation or phosphorylation as modifications, whereas the CK was found at different molecular weights, indicating cleavage of the mature protein as found in the temperature-acclimated carp (McLean et al. 2007). The sample prepared using the urea extraction protocol was run on 2DE to identify proteins affected by hypoxia. Six proteins were found to be significantly altered, one protein showing an increase in abundance whereas the remaining five were decreased. Unfortunately, these proteins were deemed too faint for identification by MS. The very subtle effects of hypoxia in zebrafish muscle was in contrast to the transcriptional studies carried out on carp and larval zebrafish (Gracey et al. 2001;

Ton et al. 2003), where a large number of mRNAs were found to be modulated. The observed difference in response is likely to be a reflection of the larval zebrafish (Ton et al. 2003) being whole animals with multiple organs present as opposed to muscle alone. Differential tissue response to hypoxia has been observed in zebrafish (Roesner et al. 2006), especially between heart and brain. In addition, there may also be issues of the later development of the circulatory system between larval and adult fish. These results indicate that alternate strategies to cope with hypoxia at different life cycle stages may exist.

Disease Responses

One of the most intensely studied aspects of fish biology as related to aquaculture is the response to pathogens and how the immune system of fish copes with infection. The understanding of this is vital for efficient aquaculture and development of vaccines. In addition, understanding of the fish immune system enables studies to be performed on the evolution of particular aspects of immunity and the molecules involved in these processes (Bird et al. 2006). The immune system is broadly split into the innate immune response and the adaptive immune response. The innate system (Magnadottir 2006) provides fish with a first line of defense against a pathogen, and the correct innate response needs to be elicited in order to fight off infection. Bacterial, viral, protozoal, and fungal infections all elicit a different response (Akira et al. 2006), many of which are highly conserved throughout the vertebrates. The invasive pathogen is recognized by cell receptors, and a complex series of signaling induces cells to activate response genes. These cell surface molecules, often Toll receptors, recognize pathogen-associated molecular patterns (PAMPS) (Janeway Medzhitov 2002). The majority of proteomic studies in fish relating to immune response have been performed during the early stages of infection and not related to the protection that is elicited following a vaccination or previously infected fish. One aspect of the innate immune system is the acute phase response characterized by the production of plasma proteins to defend against pathogens. Characteristically, in mammals these proteins are synthesized in the liver under the control of the cytokine interleukin 6 (Streetz et al. 2001) and secreted into the plasma where they exert their function. Many of these proteins have been studied in detail in salmonids including serum amyloid A, complement factors, antiproteases, transferrin, α-microglobulin, lectins, and haptoglobin (Holmskov et al. 1994; Jensen et al. 1997; Bayne et al. 2001b; Bayne and Gerwick 2001a; Douglas et al. 2003). Gene expression studies using EST analysis, suppression subtractive hybridization, and microarray have confirmed a large number of responsive genes mainly from immune organs such as the liver, head kidney, and gill (Ewart et al. 2005; Martin et al. 2005). Analysis of these genes gives indications of the type of immune response being elicited; however, as many of the transcribed proteins are destined to be secreted, the transcriptome does not give a clear indication of the amount of circulating protein. This is a good example highlighting how proteomics can give a true reflection of the response in a tissue, as the plasma does not transcribe or translate its own proteins.

During an acute phase response in rainbow trout, plasma proteins have been examined following inflammation. One early proteomics report (Gerwick et al. 2000) used 2DE gels of plasma samples from trout injected with or without a *Vibrio* bacterium emulsified in Freund's incomplete adjuvant. One major protein spot increased in

abundance in the infected fish was excised and subjected to partial amino acid sequence analysis and identified as a precerebellin-like protein, an increase in mRNA expression for this was also observed in liver. Further plasma proteome studies by the same authors (Gerwick et al. 2002) investigated the responses to different pathogens in rainbow trout—either bacteria, virus, or yeast. The bacterial infection was *Vibrio anguillarum* emulsified in Freund's incomplete adjuvant, the yeast infection was live bakers yeast (*Saccharomyces cerevissiae*), and the viral infection included both hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). Native proteins were separated on 1D gels and analyzed for different intensities. Although a number of protein bands were altered in abundance, only one protein was subjected to N-terminal amino acid sequencing and was shown to be haptoglobin, confirming this as an acute phase response protein. These reports demonstrated that it was possible to characterize the plasma proteins following infection, although the number of proteins being identified was limited as no MS was utilized.

More comprehensive coverage of the proteome during the acute phase response was achieved by Russell et al. (2006) where an acute inflammatory response was initiated by intraperitoneal administration of purified *Aeromonas salmonicida* lipopolysaccharide emulsified in Freund's incomplete adjuvant or a commercial oil-based multivalent vaccine. Following 2DE, proteins were stained by SYPRO ruby (Molecular probes, Eugene, OR), which is more sensitive than commonly used Coomassie blue stain. The gel analysis revealed large individual variability of protein spots with only those showing directed response of threefold change or greater included for identification. Several proteins were found to be present on the gels on more than one occasion; for example, two apolipoprotein spots were identified at 10.5 and 24.4 kDa. Two precerebellin spots at 24 kDa were increased in abundance, another increased protein was transferrin, whereas myoglobin was shown to be decreased. These results corroborated gene expression findings in Atlantic salmon, where transferrin, precerebellin, and apolipoprotein mRNAs were increased following bacterial infection (Tsoi et al. 2004; Ewart et al. 2005).

Proteomics has added to the panel of acute phase response proteins during studies on the loach (*Misgurnus anguillicaudatus*) (Wu et al. 2004) following skin injury, which can induce an acute phase response. Using 2DE followed by MS, a signal recognition protein, gastrin 71, and parvalbumin were described as novel acute phase response proteins with other well-characterized proteins such as apolipoprotein and C-reactive protein also increased.

A combination of approaches has been utilized to examine the Atlantic salmon proteome following infection with two major commercially important pathogens, IHNV and the infectious agent of bacterial kidney disease, *Renibacterium salmoninarum*, in both liver and kidney tissue (Booy et al. 2005). The experimental protocol was to use freshwater Atlantic salmon parr of 80–100 g in weight, infect groups with either IHNV or *R. salmoninarum*, and then sample either 5 days following IHNV infection or 25 days following the bacterial infection. The time of sampling reflects the stages at which the fish begin to show pathology. The two approaches used were conventional 2DE and isotope-coded affinity tag (ICAT), this being the first published report of this method in fish. The ICAT methodology is elegant and involves deriving the cysteine residues of one sample (in this case the control) with an isotopically light biotin affinity tag, whereas experimental proteins are labeled with an isotopically heavy labeled tag (^{13}C). The samples are pooled, digested with trypsin, and tagged proteins isolated via avidin affinity chromatography. Proteins are made up of peptides; therefore, after the

protein is broken up into peptides, the level of protein can be extrapolated by analysis of its peptide components. This proved to be a very efficient method for identifying proteins from salmon, and abundance of the proteins was then examined following the identification.

This work has produced the largest published proteome data set in fish disease responses to date, with a total of 1,854 peptide fragments being identified. From the four different challenges, 1,453 of which were quantified as a ratio using ICAT. As expected, a large number of the proteins found were assessed as being unaltered (less than twofold increased or decreased between infected and uninfected fish). Not all peptide fragments were from unique proteins; for example, in the *Renibacterium*-infected fish three different β-2 microglobulin peptides were identified, and quantification of these was reasonably consistent with values of between 2.1- and 1.4-fold increased. In the IHNV-challenged fish, a lysosomal cofactor was found to be downregulated on seven occasions in the kidney and on six occasions in the liver. An interesting observation is that the protein expression profile for the IHNV-infected fish showed a much larger decrease in abundance of individual proteins with 294 proteins showing more than a twofold decrease as opposed to 22 being increased in liver. Similar downregulation of proteins was seen in the kidney with 172 proteins showing a decrease and only 16 proteins showing an increase in abundance. The opposite was the case following bacterial infection with 95 proteins increased in liver and only 9 downregulated, but in kidney tissue there was almost an equal number increased and decreased (46 and 43, respectively). In the liver of the bacterial infected fish, proteins upregulated included those associated with the acute phase response and inflammation including, interleukin 8, α1-microglobulin, and β2 microglobulin. Other unexpected proteins found to be altered in the bacterial infected animals included a cellular nucleic acid binding factor, which is suggested to play a role in disease-associated inflammation and stress. A surprising finding from the liver and kidney of the bacterial infected fish was the induction of an Mx protein, which is characteristically an interferon-induced protein with antiviral properties (Leong et al. 1998); however, other studies on responses to *Renibacterium* (Grayson et al. 2002), Vibrio bacterin (Salinas et al. 2004), and *Listonella anguillarum* lipopolysaccharide (Acosta et al. 2004) showed Mx to be increased, possibly in response to inflammatory factors.

The proteomic investigations described above have focused on generalized response to immunostimulants or specific pathogens. In recent years, many immune regulatory factors including cytokines have been characterized at the molecular level and are helping in understanding progression of the immune response in fish (Bird et al. 2006). A key cytokine from rainbow trout, interferon γ, has been produced as a functional recombinant protein (Zou et al. 2005). This recombinant molecule has been used to investigate both the transcriptional response in rainbow trout cell lines by microarray (Martin et al. 2007b), where it was shown to induce genes characteristic of an interferon response. To further investigate the proteome response to this recombinant interferon, an Atlantic salmon cell line SHK-1 was chosen for a proteomics study (Martin et al. 2007a). The SHK-1 cell line had been developed from Atlantic salmon head kidney cells with a typical macrophage morphology (Dannevig et al. 1997; Koppang et al. 1999), and was chosen as it may have significant interferon γ responsiveness. The SHK-1 cells were stimulated with 20 ng/mL of the recombinant protein, a dose predetermined to give maximal response, and left for 24 hours for induction of genes and subsequent translation of the proteins. The 2DE gels were performed on quadruplicate samples of either stimulated or unstimulated control

cells. Proteins were separated on IPG strips (pH 4–7) and then on 10% acrylamide gels for the second dimension. Gel analysis revealed 22 proteins to be significantly altered in abundance. Fifteen proteins showed increased abundance with 7 decreased, 11 of these were chosen for PMF. The peptide masses generated were first compared against the NCBInr database using the MASCOT search program, and 5 proteins were found to have identity. Second, the peptide masses were used to search against nucleotide sequences for Atlantic salmon and rainbow trout that had been complied by the cGRASP consortium (http://web.uvic.ca/cbr/grasp/), where contigs of all salmon and trout EST sequences have been generated. These nucleotide databases were searched using the MS-Fit program as described earlier, and all the peptide fingerprints were found to have nucleotide sequences that produced matching peptides. One protein spot was identified differently by each program, a human MHC I antigen by MASCOT, whereas using MS-Fit it was shown to be a nucleosome assembly protein. To ascertain definitively the identity of this protein, LC MS/MS was performed on the trypsin digest products. These fragments were used to search the NCBInr database and on this occasion a significant hit was obtained against a zebrafish nucleosome assembly protein confirming this to be the correct identity. Real-time PCR analysis was used to assess the correlation between the mRNA and protein abundance for a subset of the identified proteins. Generally, there was a parallel change in expression but at different magnitudes between the protein and mRNA. One protein, glucose-regulated protein 78 (GRP78) (Stoeckle et al. 1988), which was increased in abundance as protein did not show greater mRNA expression, indicating there may be different mechanisms regulating the abundance of the protein. The mRNA encoding GRP78 is known to be responsive to various factors including heat shock (Ojima et al. 2005) and following a bacterial infection (Martin et al. 2006), although in these studies no protein abundance was measured.

Nutrition and Growth

Although growth is determined by a multitude of factors, including temperature, health status, genetics, and water quality, to obtain maximal growth in aquaculture nutrition is a key feature. Indeed, up to 65% of the cost of aquaculture production of carnivorous fish is on feedstuffs. Protein deposition is key to muscle growth and has been extensively studied at the whole animal level (Houlihan et al. 1995; Ballantyne 2001; Carter et al. 2001). The regulation of protein metabolism is central to understanding how efficiently fish deposit their ingested protein as growth. Once a fish ingests protein, it is proteolytically digested and absorbed as peptides and free amino acids, which are then added to the free amino acid pool for continual synthesis and turnover of proteins within the fish. There is a correlation between protein consumption and protein synthesis, but this does not lead to greater protein growth within groups of fish eating the same amount of food (Houlihan et al. 1995); hence, the rate of protein degradation may be important when considering the efficiency of deposition of ingested proteins.

To initiate proteomic studies on the mechanisms of protein degradation in rainbow trout, fish were subjected to 2 weeks of food withdrawal (Martin et al. 2001). This was expected to alter the balance between protein synthesis and degradation, with a shift toward greater protein degradation as fish begin to catabolize their own tissues. In parallel, lipid mobilization would be altered among other metabolic processes. The

trout lost weight at 0.34% per day in comparison to the fed group that gained 1.6% per day. Proteome analysis of liver tissue found 24 proteins that were significantly altered because of the food withdrawal. A number of proteins were subjected to PMF. At the time of this work (November 2000), there were only a limited number of rainbow trout protein sequences in the NCBInr database, as the large EST programs in salmonid fish (already mentioned) had only just begun; so, the number of proteins identified was limited. One protein that increased in abundance after fasting was cathepsin D, a major lysosomal protease involved in cellular protein degradation. Northern blot analysis confirmed the mRNA for cathepsin D was also increased during this short-term starvation. Activity of cathepsin D has been shown to be increased in both liver and muscle tissue of migrating pacific salmon when they undergo voluntary starvation during the spawning migration (Mommsen 2004). Although the natural fasting found in salmon is much more extensive than what is experienced by rainbow trout. Other reports also have shown increased transcriptional activity for cathepsins D, L, and S in atrophying muscle of rainbow trout (Salem et al. 2006b). The other key protein degradation route, the ubiquitin proteasome pathway, is known to be altered between fed and starved fish (Martin et al. 2002; Salem et al. 2006a); however, no protein spots were identified in the liver during this experiment possibly due to lack of sequence coverage at the time. These results may give indications of the state of protein metabolism in growing fish. Measurement of synthesis rates within whole fish has been possible by various methods (Houlihan et al. 1995); however, measurement of individual proteins is still problematic and has not been achieved successfully in fish. Recently, methods have been developed in chicken to determine synthesis rates of single proteins in vivo (Doherty et al. 2005; Doherty and Beynon 2006), but this has yet to be adapted to studies on fish.

An understanding of protein metabolism and its relationship to growth and health is extremely important when formulation of diets is considered. The global supply of fishmeal and fish oils will become limited, as the capture of wild fish for fishmeal and fish oil production has reached plateau levels (Naylor et al. 2000). Currently, there is a necessity to improve the utilization of the current feeds and to also replace the fishmeal and fish oils with proteins and oils derived from sustainable plant products. In addition, reduction in fish oil levels in feeds may reduce the contamination of marine foodstuffs with pollutants such as PCBs and dioxins, which eventually become deposited in the oil-rich tissues of farmed fish (Berntssen et al. 2005; Bethune et al. 2006). Carnivorous fish that include the salmonids, sea bass, and sea bream require fishmeal (fish-derived protein) and fish oils in their diet with only a limited requirement for carbohydrate. It is now possible to replace to a high proportion of fishmeal (protein) in aquaculture feeds with plant-derived proteins (Benedito-Palosa et al. 2007; Wilson et al. 2007), but there is still large variation in how the fish respond to these diets. Imperative to use plant proteins is to ensure the correct balance of essential amino acids is available. Proteomic studies on the liver of rainbow trout have centered around either partial or complete replacement of fishmeal proteins with plant-derived proteins (Martin et al. 2003; Vilhelmsson et al. 2004). During these experiments, fish were maintained for 12-week growth trials during which growth, protein deposition, whole animal protein synthesis, and free amino acid levels were measured. In the first experiment, fish were maintained on either normal fishmeal or partial plant protein mixture with the major plant protein being soy (Martin et al. 2003). Those on the plant protein diet had significantly higher protein synthesis rates, but not higher

growth rates indicating poor efficiency of protein deposition. The 2DE proteomics on the liver identified a panel of 33 proteins significantly altered in abundance, of these 23 were chosen for identification by PMF and MALDI-TOF MS. The peptide fragments generated were screened against both the NCBInr database and the available salmonid ESTs using MS-Fit, and from this analysis 17 proteins were assigned an identity. Among the proteins identified were several heat shock proteins (HSP70, HSP108, and GRP78). HSP70 was found to be downregulated in the liver of fish fed the soymeal-containing diet. Two different protein spots were identified as HSP108 (also described as a transferrin-binding protein) found at 54 and 79 kDa. In the fish fed the soymeal diet, the 54-kDa isoform was the major one observed, whereas the 79 kDa was predominant in the fishmeal-fed group. A third HSP, GRP78, was found to be upregulated in the fish fed the soymeal diet. This protein as described earlier may have immune function properties, and it could therefore be inferred that immune function was affected by this diet. Together this may suggest that there are alterations in the protein metabolism in the livers of these fish as shown by the alterations in protein chaperones, indicating there is a stress response to components of the soy protein. In addition, two Apo-AI proteins were found, with one of these being significantly downregulated in the soymeal-fed fish and the other not being affected. Apolipoprotein is the major plasma high-density lipoprotein and is a potent activator of the enzyme lecithin/cholesterol acyl transferase, which mediates the removal of free cholesterol to the liver for excretion (Brouillette et al. 2001). It is most likely that these two spots are related to a posttranslational modification by cleavage, as they are found at different molecular weights. In mammals, phytoestrogens in soy increase Apo-AI expression (Ribeiro et al. 1991), while other factors in soy may decrease its expression (Lamon-Fava et al. 1999). The implications of this are that the soymeal used in these diets was also affecting the cholesterol metabolism of the fish. These factors together would imply that cofactors and/or antinutritional factors (ANFs) were copurified and not removed from the soy proteins, resulting in stress and dysfunction of metabolic pathways in the liver. Subsequently, further new diets were formulated with plant proteins derived from a variety of plants including maize, wheat, peas, and rapeseed (Vilhelmsson et al. 2004), and again the fish fed the plant proteins grew slower than those fed fishmeal diets. Proteomic analysis found many of the proteins altered in expression were related to energy metabolism indicating that on this diet the fish had higher energy demands as shown by enzymes including aldolase B, malate dehydrogenase, and electron transfer flavoprotein α subunit. Clearly from these studies although fish can perform reasonably well on diets containing plant-derived proteins, copurified ANFs may still cause a problem to the fish, but on this occasion not resulting in a stress (heat shock) response. This problem can be overcome by extended treatments of the diets and choosing those plant sources that result in less carry over of ANFs (Gatlin et al. 2007); however, this increases the cost, so at present complete plant protein diets are not commercially viable for aquaculture.

Early Development

Correct early development is essential for efficient aquaculture. As the embryo develops within the egg, maternal mRNAs are used in parallel to embryonic mRNAs. For these reasons, both the state of the ovary during oogenesis and early time

postfertilization are key for the correct cascade of genes and protein synthesis for development. If incorrect or disturbed, it can lead to a multitude of problems. Although not an aquacultured species, the zebrafish is an extremely important tool for studying fish biology (as reviewed in Chapter 7) and may also be used as a model for vertebrate development due to its short life span, ease of maintenance, and large amount of genomic information. The zebrafish does not lend itself to proteomics easily as the tissues are usually very small and eggs/larvae even smaller. In order to examine the proteome of developing zebrafish, large numbers of embryos are sampled at fixed times postfertilization providing a sufficient quantity of material to carry out 2DE. This approach has been used to examine the proteome of larvae from 6 hours postfertilization until they were 1-week old (Tay et al. 2006). The initial stages up to 18 hours showed little change in the proteome, but then at later stages, most likely as organogenesis began, the proteome changed dramatically. Between 18 and 24 hours postfertilization, the number of large proteins above 50 kDa decreased with a parallel increase in small proteins in and around 25 kDa. The changing profiles could be the result of several factors, primarily that the new smaller proteins could reflect the morphological changes linked to the developing organs. Alternatively, high-molecular-weight proteins may be cleaved to produce functional proteins of a lower molecular weight. One hundred eight proteins were subjected to MALDI-TOF/TOF MS that matched 55 different genes, 20% of these had more than one protein spot, similar to what has been observed in many proteome studies. The identified proteins were from diverse functional groups as would be expected; however, even though the embryos in this study were deyolked, a large number of vitellogenin and related proteins were identified. These proteins may represent some of the smaller proteins derived from precursors mentioned above. The appearance of large numbers of vitellogenins and their cleavage products was also the case in other developmental studies in zebrafish (Link et al. 2006b) and in a rainbow trout study (Kanaya et al. 2000), where almost all the proteins identified from 2DE gels were vitellogenin fragments. In addition, vitellogenin and one of its cleavage products lipovitellin was observed to accumulate in the coelomic fluid of rainbow trout during a study (Rime et al. 2004) on postovulatory aging of eggs. This has potential as an indicator of egg quality as developmental success decreases the longer the delay between ovulation and fertilization.

A study on the regulators of germ layer morphogenesis in zebrafish (Link et al. 2006a) found 37 significantly regulated proteins by 2DE. Following LC MS/MS, 35 of these were identified as specific proteins, many of which were involved in cytoskeleton organization. A parallel microarray experiment was performed on the same tissues that revealed no significant overlap between the proteomic expression pattern and the gene expression patterns generated by microarray, indicating that there is significant translational or posttranslational regulation of proteins at the early stages of development.

Use of Cell Lines in Fish Proteomics

Cell lines offer many advantages over in vivo whole tissue or primary cell cultures, as the cell line population is not mixed as occurs in primary culture nor is there animal-to-animal variation. Different salmonid cell lines have been assessed for their

use in proteomics (Wagg and Lee 2005), where a number of the key rainbow trout and Chinook salmon cell lines were examined for both morphology and proteome. The similarity of cell lines from rainbow trout ranged from 33 to 47%, whereas from salmonid to nonsalmonid, for example, Chinook salmon embryo cells to goldfish epithelial cells showed a protein correlation of between 0 and 22%. None of these cell lines appear similar to the SHK-1 Atlantic salmon cell line described earlier (Martin et al. 2007a). The great difference between these cell lines was surprising. Unfortunately, this report did not make any attempt to identify individual proteins, and as such these correlations may be an overestimate where protein spots of similar pI and molecular weight may not necessarily be the same protein. It would, however, be interesting to compare the cell line with primary cultures from the source organ, but to date this has not been published for fish cells.

Calibration with Transcriptome

Transcriptomics and proteomics both attempt to describe the functioning of the cell and how this can then lead to the phenotype of an animal. Currently, well-characterized microarray platforms are being developed for all areas of life sciences including those relating to the aquaculture industry. As more proteome studies are reported, it is clear that there is not a simple correlation of mRNA to protein spot as shown on 2DE gels. This reflects the posttranslation processing of proteins by many different mechanisms; hence, the proteomic approach will help interpret the transcriptome information. To date, there are no comprehensive comparisons made between microarray analysis and proteome of aquacultured fish, but the zebra fish study (Link et al. 2006b) is an example of a fish investigation, which showed only limited correlation. Studies on mammalian liver were the first comprehensive microarray versus liver analysis (Anderson and Seilhamer 1997) and showed poor correlation of expression between the two approaches. Since this time, many other tissue (Scheurer et al. 2004) and cell lines (Conradas et al. 2005; Kuo et al. 2005) among others have all shown similar lack of correlation. As well as posttranslation modifications, differences in transcriptional and translational regulation can lead to discrepancies between the level of expressed gene and protein activity. The rate of translation and half life of the proteins is not revealed by standard proteomic methods. This area of research is being addressed and has many additional technical issues that include stable isotope metabolic labeling of cells in either culture or intact animals (Doherty et al. 2005).

Future Perspectives

The use of proteomics within aquaculture is still in its infancy, but has advanced dramatically in recent years. Without the DNA sequence data none of this would have been possible and in fish, the generated sequences are making the identification of proteins much more reliable. Although many contigs have been generated from EST data sets, many of these do not represent full coding regions of the transcripts and it will still require complete genomes to be sequenced for proteins to be reliably identified as in yeast and mice. As bioinformatics improves and genes and proteins become better

annotated for gene ontology, a much clearer picture will emerge as to the biological and functional processes that are being modulated in specific experiments. The future technological developments will include protein chips (Lubomirski et al. 2007), which now appear a long way in the future for fish but such products are already for sale for human protein families.

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Chapter 7
The Role of Model Organisms in Aquaculture Research: Transient and Permanent Advantages

Barrie Robison

Aquaculture and Biotechnology

The goal of aquaculture is to culture fish species for consumption and ultimately earn profit. While the profitability component of aquaculture is better addressed by economists, there are several challenges facing the aquaculture industry that are effectively addressed with genomics and biotechnology. These include (but are not limited to) analyzing the genetic basis of complex traits; gaining an increased knowledge of metabolism, growth, and protein turnover; characterizing physiological responses to the environment and perturbations thereof; and determining optimal nutrition from alternative diets. In addition, the fact that wild progenitor populations are available for most cultured fish species allows potential introgression of desirable traits from wild populations into cultured stocks. This invites investigations into the genetic basis of domestication itself.

In this chapter, we consider the role of teleost model organisms in aquaculture research, mainly focusing on studies done in zebrafish. We argue that there are several critical research areas relevant to aquaculture that could benefit from comparatives studies using both model and cultured species. These research areas include the genetic basis of complex traits, the physiological response to environmental perturbations, nutrient utilization, and the process of domestication.

Genetic Basis of Complex Traits

One of the most obvious ways in which biotechnology can improve profitability in aquaculture is by accelerating genetic improvement of cultured stocks. However, to move genetic improvement beyond more traditional selection on desirable traits, detailed knowledge of the genetic basis of specific economically desirable traits is required. For the most part, these economically desirable traits have a quantitative genetic basis, meaning they are highly polygenic and often are affected by both genetic and environmental variance (Davis and Hetzel 2000).

There are many examples of traits that have been analyzed genetically in aquaculture species, including growth characteristics (Thorgaard et al. 2002; Gall and Neira 2004; Drew 2007), performance (Jackson et al. 2002; Overturf et al. 2003), disease resistance (Nichols et al. 2004; Barroso et al. 2008), and behavior (Gadagkar and Doyle 1999). The identification of the genetic basis of complex traits is often very

challenging. This challenge is frequently the result of difficulties in transitioning from the identification of quantitative trait loci (QTL) to positional cloning of candidate genes.

In this chapter, we consider the potential role for model teleosts in identification of the genetic basis of complex traits that are relevant to the aquaculture industry. The use of model organisms in this regard is somewhat analogous to the use of model organisms in biomedical research, and there are several areas of research in which model teleost species can facilitate research into the biology of cultured fish species.

The Physiological Response to the Environment

The successful culture of aquatic organisms depends critically on optimal water quality. Susceptibility of cultured fish species to perturbations in water quality and other environmental variabilities directly affects profitability. It is therefore critical that culturists and researchers alike understand the physiological responses of cultured fish species to environmental perturbations and the underlying genomic mechanisms that regulate these responses. This knowledge will assist in (1) identification of the key environmental variables that may impact cultured species and (2) design of effective mitigation measures to prevent losses and treat impacted animals. Related research in aquaculture species varies from improving the ability of tilapia to spawn in brackish water (El-Sayed et al. 2003) to improving the heat tolerance in salmonids (Somorjai et al. 2003).

Optimal Nutrition Requirements

A better understanding of the physiology, immunology, and metabolic pathways of teleost fish will allow producers to use more efficient means for optimizing the growth of commercially important species. Improved comprehension of nutrient partitioning in the animal provides nutritionists the information to formulate complete diets that match the animal's energy, vitamin, mineral, and specific amino acid needs. Better-formulated feeds will also be utilized more efficiently, thus reducing wasted material and inefficient dietary components. Research is actively pursuing improved diet formulations from sustainable products for many species (Gatlin et al. 2007). Finally, enhanced knowledge of the behavior and immunological systems of teleost fish will enable aquaculturists to manage stocks at higher densities while minimizing losses due to disease.

Domestication

Many cultured fish species are still in the process of becoming domesticated. In fact, wild progenitor populations are available for most cultured stocks. This presents both opportunities and challenges. In terms of opportunities, the substantial variation present in many aquaculture species may allow geneticists to introgress desirable traits, including disease resistance, improved stress physiology, or even enhanced capabilities for dietary utilization into species broodstock. However, the potential

impact of cultured stocks on existing wild populations remains a concern (Naylor et al. 2000). In addition, the ongoing adaptation of many cultured stocks to the culture environment provides an opportunity to study the genetic changes that occur during domestication (Ruzzante 1994; Fleming et al. 2002).

The Role of Model Organisms

Model organisms have played a critical role in biomedical research, and significant amounts of funding from the National Institutes of Health are targeted toward fruit flies, nematodes, and even yeast. The underlying rationale behind this investment is that there are fundamental biological processes, such as cellular metabolism, aging, transcription, and translation, that are common to all eukaryotes. Many diseases arise from defects in these processes, and researchers can learn a tremendous amount from these seemingly "lower" organisms. In addition, a critical feature of the model organism approach in biomedical research is the fact that certain experiments simply cannot be performed in humans. In many ways, humans make a tremendously bad research subject for genetics; we have long generation times, small family sizes, and tend to resist attempts at designed matings. For this reason, basic research in model organisms has generated a wealth of knowledge that has been applied to understanding complex systems in humans.

A related approach can also be used to gain a better understanding of specific genetic and physiological processes in aquaculture species. This is certainly not a perfect parallel, as we can perform experiments in rainbow trout and catfish that we could never dream of doing in humans. However, model teleost species do have significant logistical (space, time) and infrastructure (primarily genomic tools) advantages relative to aquaculture species. Similar to biomedical research in flies, worms, and even mice, our challenge becomes one of *translation*. Instead of translating knowledge from laboratory bench to the hospital bedside, researchers must translate knowledge from the laboratory to the raceway, pond, and fish farm. Unlike biomedical research, however, our motivation in translating research from model teleosts is one of economics, as aquaculture is focused on production and profit.

Logistical Advantages of Teleost Models

There are obvious logistical advantages to using model organisms for genetic research. Foremost among these are short generation times and relatively forgiving space requirements. For example, genetic experiments such as QTL analyses often require crosses that span three or more generations. Considering that zebrafish have a generation time of roughly 3 months, one could create a panel of 1,000 second-generation (F2) fish in less than 1 year (Bilotta et al. 1999). Even if rainbow trout had the same set of genomic tools as the zebrafish, similar F2 designs in rainbow trout would take 3 years to create sexually mature F1s. And if the traits under consideration were only measurable in adults, then it would take 1–3 more years before phenotypic data were collected. Model teleost species also have considerable advantages in terms of space requirements and research costs. Teleost models are usually small, take less tank space and water, require less food, and often are less labor intensive to maintain.

Thus, experiments using large numbers of individuals across multiple generations are both faster and less expensive.

The logistical advantages of model teleosts for genetic research will never go away, unlike the relatively transient advantages of available genomics infrastructure described below. In an ideal world, one could pose a question regarding the genetic basis of a desirable trait, and given sufficient financial resources, one could rapidly arrive at an answer in a model teleost. This information could then be used to frame hypotheses about the mechanism in closely related aquaculture species, "leapfrogging" some of the cumbersome steps of genetic designs in long-lived species.

However, this approach makes a critical assumption: that the mechanisms regulating the trait in question are conserved between the model and the cultured species. Considering the massive amount of diversity within the teleost lineage, this assumption is somewhat questionable for certain traits, such as energy utilization of specific nutrients. In fact, for some traits with highly polygenic modes of inheritance, the underlying genetic basis may not be the same among crosses within a species, let alone between species (Wittbrodt et al. 1998). This is not problematic from an academic standpoint, as something can still be learned in regard to the comparative evolutionary trajectories of two teleost lineages. Ultimately, increased basic understanding of metabolic pathways and nutrient utilization will allow researchers to better design future experiments that directly address applicable problems in aquaculture.

Genetic Advantages of Teleost Models

At present, there are considerable advantages of using model fish species for genetic analysis. For several fish species, there is already an extensive genomics infrastructure and a toolset in place that allows researchers to rapidly answer genetic questions. These tools can be roughly categorized into analysis methods associated with the genome, the transcriptome, and the proteome.

Genomic tools available in model species include complete reference sequences for the genome, detailed lists of polymorphisms such as single-nucleotide polymorphisms and microsatellites, and other tools such as available mutants and TILLING (*t*argeting-*i*nduced *l*ocal *l*esions *in g*enomes) approaches. The availability of these tools is predicated on the fact that for many models the genetic precursors of modern-day genomics were developed more than a decade ago, including genetic maps, physical maps, and large insert libraries.

Interestingly, cutting-edge genomic tools are now either developed or being pursued for most economically important cultured fish species. Although aquaculture species will lag behind traditional models for the next few years, it seems obvious that we are on the doorstep of an era where every species has the potential to be a genetic model organism. New advances in sequencing by synthesis methodology (454 and Solexa) indicate that genome sequences for most aquaculture species will be available soon. Advanced genotyping methodologies also seem readily adaptable to cultured species, though the effectiveness of many of these technologies depends on a sequenced reference genome for full utilization. Microarrays for some aquaculture species are already available (Rise et al. 2004; Li et al. 2007; Salem et al. 2008), and new methods such as direct Solexa sequencing of the transcriptome may make structured arrays a thing of the past (again, the utilization of these technologies is

best accomplished using a genome sequence). Thus, although the current state of affairs indicates an advantage in terms of genomic infrastructure to teleost models, this technical advantage is likely to wane over the next 5–10 years.

Teleost Models

Zebrafish

Zebrafish (*Danio rerio*) (Figure 7.1) are small minnows native to the rivers of Pakistan and India (You and Korzh 2005) and, under laboratory conditions, are reproductively active throughout the year (Eaton and Farley 1974; Bilotta et al. 1999). Originally, advocated as a model system by Streisenger in the late 1970s (Streisinger et al. 1981), zebrafish have become a prominent model system for the study of developmental biology and genetics. They are easily maintained in the laboratory, allowing large numbers to be housed together. Generation time is short relative to other vertebrate model organisms and a single spawning produces large numbers of eggs (Eaton and Farley 1974). There is also an extensive genetic toolbox available for the zebrafish, including detailed genetic maps (Woods et al. 2005; Phillips et al. 2006; Bradley et al. 2007), libraries of pregenerated mutants (Dooley and Zon 2000), DNA microarrays (Ton et al. 2002; Mathavan et al. 2005), and a nearly complete genome sequence (http://www.sanger.ac.uk/Projects/D_rerio/). Genetic analyses have aided in the discovery of zebrafish homologs of humans genes (Barut and Zon 2000; Blake et al. 2000). Zebrafish are very amenable to forward genetic screens (Muto et al. 2005) and tolerate chromosome set manipulation (Postlethwait et al. 2000). Mutants are easily produced and have allowed researchers to investigate developmental and behavioral abnormalities (Brockerhoff et al. 1995; Chen et al. 1996), though behavioral abnormalities are usually investigated from a developmental standpoint (Kokel and Peterson 2008). The use of antisense RNA for knocking down the expression of specific genes has been avidly pursued using zebrafish and yielded a wealth of information regarding individual gene activity (Nasevicius and Ekker 2000).

The proliferation of zebrafish research and its establishment as a model organism has been driven primarily by biomedical research. The National Institutes of Health has long recognized the utility of the zebrafish in experimental studies, as indicated by the number of projects that have been funded and the amount of funds allocated to those projects: a tenfold increase in the number of projects funded from 1992 to 2003,

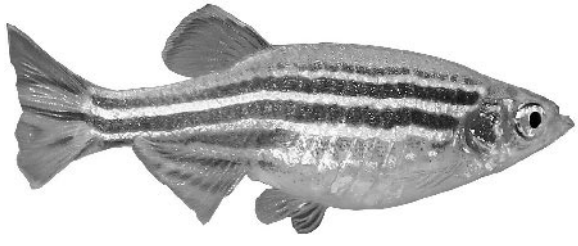

Figure 7.1. Zebrafish (*Danio rerio*).

with 2003 funding reaching $61 million compared to a little more than $10 million in 1992 (Rasooly et al. 2003).

Medaka

The medaka (*Oryzias latipes*) (Figure 7.2), also known as the Japanese killifish, is an ornamental fish from Southeast Asia. A high-quality draft sequence of the medaka genome was recently published (Kashara et al. 2007). There are several available genomic databases for medaka, including those for expressed sequence tags (ESTs) (Kimura et al. 2004), information on toxicological studies (Villalobos et al. 2000), expression patterns, and cDNA and genomic sequences. The medaka genome is less than half the size of the zebrafish genome, and new studies have identified approximately 2,900 previously unannotated genes (Semon and Wolfe 2007). Considering the variations found between fish species, newly identified genes can provide a wealth of information regarding the impact of specific genes on phenotype and physiology.

The medaka is used as a model organism in evolutionary studies, and comparative studies between medaka and zebrafish illustrate several of the conserved genetic and molecular mechanisms involved in vertebrate development (Furutani-Seiki and Wittbrodt 2004). A great deal of research has also been done in medaka regarding the genes that play a role in organ development (Furutani-Seiki et al. 2004). Transgenic modification is also relatively easy with medaka and a significant number of mutants have been generated including fish whose cells express fluorescent tags for use in development studies, *cll* mutants for toxicology studies, and phytase expressing mutants that demonstrate an enhanced ability to digest phytase phosphorous (Hostetler et al. 2005; Kinoshita et al. 2008).

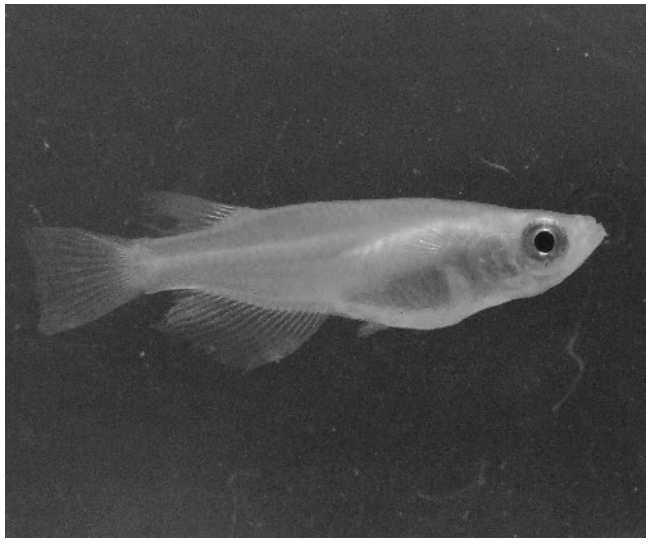

Figure 7.2. Medaka (*Oryzias latipes*).

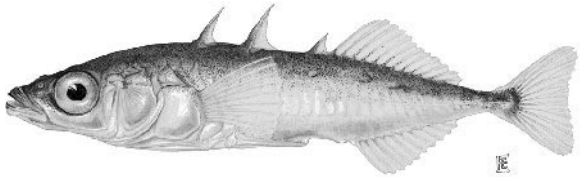

Figure 7.3. Threespine stickleback (*Gasterosteus aculeatus*).

Stickleback

Another species with potential for serving as a model teleost for aquaculture research is the threespine stickleback (*Gasterosteus aculeatus*) (Figure 7.3). These fish are native to most of the Northern Hemisphere and are of great interest to evolutionary biologists because of the diversity in morphology found within this genus and because of instances of parallel evolution of morphological traits (Cresko et al. 2007). The ancestral sticklebacks exist almost exclusively in marine ecosystems and maintain a homogeneous appearance with similar behaviors and life histories. However, as recently as 12,000 years ago sticklebacks became trapped in glacial-formed systems and have since evolved to survive in many types of diverse freshwater systems; this diversity is reflected in their varied morphology (Peichel 2005).

The genome for the stickleback is sequenced and several libraries have been made from tissue samples of fish from many different environments. Other molecular tools available for stickleback studies include thousands of ESTs from different tissues, which are available from GenBank (Kingsley et al. 2004), and the development of transgenic modification technologies, which are proving useful in various studies (Colosimo et al. 2005). Much of the work involved with the stickleback involves studies evaluating how these organisms quickly responded to changes in their environment and how these changes are represented on a genomic basis (Peichel 2005). Furthermore, several current studies are addressing whether these genetic changes are similar for animals found in geographically separate but similar environments. Natural variation in sticklebacks has been mapped using restriction site-associated DNA (RAD). The use of RAD tags was first tested in stickleback to demonstrate the applicability of RAD markers for individual and bulk genotyping (Miller et al. 2007).

Fugu

The fugu (*Fugu rubripes*) (Figure 7.4) is a small Japanese pufferfish that possesses an unusually small genome that contains relatively little nonexpressed genomic

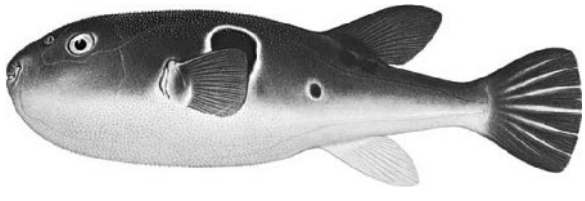

Figure 7.4. Japanese pufferfish (*Fugu rubripes*).

sequences. The 400-Mb genome of the fugu fish is approximately eight times smaller than the genome of humans but expresses a similar number of genes (Brenner et al. 1993). Because of its small genome size, the fugu fish was the first fully sequenced teleost genome. This fish therefore makes an ideal model for studies involving the identification of conserved functional elements. *Tetraodon nigroviridis* is another Asian pufferfish that actually possesses the smallest sized genome of approximately 340 Mb (Crollius et al. 2000). The genomes of both of these fish have been sequenced and are used in comparative genomic studies (Venkatesh and Yap 2004).

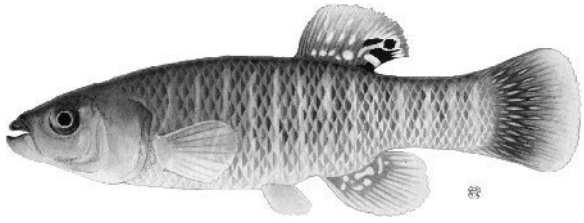

Figure 7.5. Mummichog (*Fundulus heteroclitus*).

Fundulus

The mummichog (*Fundulus heteroclitus*) (Figure 7.5) has been used in significant research studies dating as far back as the late 1900s by researchers at Woods Hole, MA. This fish is present in the eastern USA and can be found in environments that vary greatly in temperature and water salinity (Fritz et al. 1975). Many initial experiments were conducted using mummichog for studies in genetics, pigmentation, and endocrinology. Because the mummichog has been used as a research animal for such a long time and so much is known about its physiology, it logically progressed into a primary research organism. As with other fish species that have been studied for a significant period of time and whose physiology is well known, the mummichog was and is used significantly in toxicology and environmental studies (Baumann 1998; Hiramatsu et al. 2006; Burnett et al. 2007). Currently, the genome of this species has not been completely sequenced; however, the genomic tools available for this species includes a large collection of EST libraries and cDNAs, developed microarrays for gene expression studies, transgenic and knockout mutants, morpholinos, and clones (Kirchoff et al. 1999; Burnett et al. 2007).

Fathead Minnow

The fathead minnow (*Pimephales promelas*) (Figure 7.6) is a temperate freshwater cyprinid that is found throughout much of North America. It is also listed as an invasive species in Europe. It has been used in research including toxicogenomics and environmental toxicology and as a biomonitor for detecting the presence and effects of toxic compounds (Ankley and Villeneuve 2006). A searchable toxicity database developed from studies carried out with this species is available (http://www.epa.gov/ncct/dsstox/sdf_epafhmlthml). Compared to other research

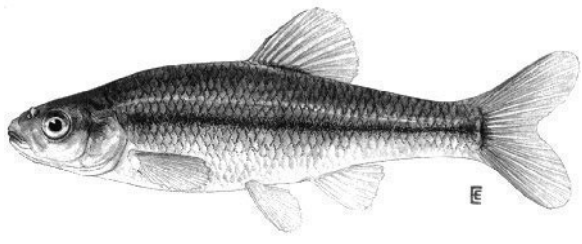

Figure 7.6. Fathead minnow (*Pimephales promelas*).

teleosts, genomic tools for the fathead minnow are more limited. The genome has not been completely sequenced yet; however, the development of ESTs from cDNA libraries obtained from multiple tissues has led to some comparative expression studies (Kane et al. 2008). Quantitative polymerase chain reaction assays have also been developed for this species as a model for endocrine disruptor research (Villeneuve et al. 2007). Furthermore, the genome of this cyprinid has been used to assist in identifying and annotating previously unidentified genes in zebrafish (Christoffels et al. 2006).

The Intersection of Aquaculture and Teleost Models

Genetics of Complex Traits

Although there is considerable potential for using models such as the zebrafish to study the genetics of complex traits, there has been surprisingly little research in this area. In aquaculture, the genetics of complex traits are typically pursued in a quantitative genetics framework, where researchers draw inferences regarding parameters such as heritability and genetic correlations (Davis and Hetzel 2000; Henryon et al. 2002). Detailed genetic analyses are typically conducted by searching for QTL. Quantitative genetic analyses in zebrafish are relatively rare. However, Wright et al. (2003) estimated a heritability of 0.2 for shoaling behavior, although this estimate had relatively large standard errors. Without specific estimates of heritability, researchers can still infer that there is genetic variation in a variety of traits that are relevant in aquaculture. For example, Robison and Rowland (2005) showed using a common garden experiment that zebrafish strains vary in both behavior and growth rate. These interstrain differences were maintained even when the strains were raised in the same tank, arguing strongly that these differences have a significant genetic component. Wright and Krause (2006) demonstrated that wild caught zebrafish strains harbored considerable variation in boldness and shoaling behavior. Similarly, Oswald and Robison (2008) showed that considerable variation exists among zebrafish strains in foraging behavior, specifically latency to begin feeding.

The source of the variation among zebrafish strains could be a result of preexisting strain variation among wild populations (e.g., Wright et al. 2003), arising as a result of local adaptation. However, there are also clear and repeatable differences in behavior among wild strains and those reared in the laboratory for many generations (30 generations or more). Thus, it is possible that at least part of the variation in growth,

feeding, and place preference behaviors (Robison and Rowland 2005; Wright et al. 2006; Oswald and Robison 2008) may be a result of adaptation to captivity. Adaptation to captivity is highly relevant to the aquaculture industry, as wild progenitor populations still exist for many species. While in conservation biology adaptation to captivity is viewed as detrimental, in aquaculture, culturists seek to facilitate adaptation to the hatchery environment. Thus, knowledge of the genetic changes that underlie this process is advantageous.

Relatively few QTL studies have been carried out in model teleost species. In this regard, research in aquaculture species is considerably more advanced. In zebrafish, Wright et al. (2006, 2007) detected QTL for both behavior- and growth-related traits in an F2 cross of a wild and a domesticated strain of zebrafish. These researchers also showed that epistatic effects played a significant role in determining behavior (Wright et al. 2007). Most research on the genetic basis of phenotypic variation in model teleosts has to date been focused on genes of large effect. Although it is tempting to regard the research of developmental mutations as influencing only Mendelian traits, many mutations have been identified that influence continuous traits, such as behavior. However, the effects of these mutations are categorical, and are not considered further.

Considerable knowledge can be gained by pursuing QTL studies of trait variation in the zebrafish. This animal has significant logistical advantages over most aquaculture species that facilitate QTL analyses. First, the generation time of the zebrafish means that a QTL experiment can be completed in less than 1 year, greatly accelerating the gain of knowledge. Second, the zebrafish harbors trait variation that is directly relevant to aquaculture, such as variation in growth traits (Chapter 11) and behavioral traits relevant to the domestication process. Third, the availability of a genome sequence and a robust genomic toolset make the positional cloning of QTL more approachable. However, it is important to note that this process is challenging even in "supermodels," such as mice and *Drosophila*.

By pursuing QTL studies in the zebrafish, the genetic architecture of traits relevant to the aquaculture industry can be determined. The next challenge is one of translation. How conserved are the mechanisms governing these traits? Detractors of a model organism approach would argue that variation in growth rate is likely to be highly polygenic and that any genes identified in a QTL study of zebrafish are not likely to generalize to a different cross of zebrafish, let alone a different species. While this is partially true, the information gathered in these studies will eventually allow researchers to build an understanding of the pathways that typically harbor interesting variation, identify novel genes that may not affect the trait in better understood systems (such as mammals), and identify conserved features typical of all teleosts. In fact, translation of the results of genetic analyses is facilitated by a comparative approach. This will ultimately benefit the industry, because it means that discoveries in one aquaculture species may be then applied to other species if the data from model organisms indicate conservation across the teleost lineage.

Physiological Response to Environmental Perturbations

Environmental perturbations are highly relevant in the context of cultured fish species. The zebrafish has seen considerable use as a toxicological model, and therefore could

be used to study physiological and genomic responses to environmental perturbations. In fact, the zebrafish is a model system that is well established in this field. The advantages of the zebrafish in this field are numerous and include the usual litany of logistical features (small size, inexpensive, rapid, and repeatable breeding). However, the detailed knowledge of zebrafish development, coupled with their optically clear embryos, allows the development of toxicological endpoints (Hill et al. 2005). Although the toxicology field often focuses on chemical toxicity that may only partially overlap with the environmental perturbations relevant to aquaculture, the biological lessons learned may be more broadly applicable. For example, environmental estrogens are a particularly important contaminant that can arise from urban effluent (Sonnenschein and Soto 1998). Xenoestrogens, chemicals that mimic the effects of estrogen in biological systems, are also a significant problem that can influence sex ratios and secondary sexual characteristics (Nimrod and Benson 1996). In addition, there is some evidence that these compounds may cause long-lasting epigenetic changes (Veeramachaneni 2008). The long-term effects of these compounds on fish abundance and population persistence are not well understood.

The utility of the zebrafish in toxicological research goes well beyond the study of endocrine disruption. In a recent review, Hill et al. (2005) provide a large list of studies examining the physiological, genomic, and developmental effects of many classes of toxins, including metals, polychlorinated biphenyls, and other pesticides and herbicides. All these environmental toxins are directly relevant to the aquaculture industry, and many are known to have deleterious effects on cultured fishes.

While the zebrafish certainly has considerable utility in identifying the mechanisms underlying toxicological responses, it is by no means a perfect model. In fact, one disadvantage of the zebrafish system in this regard is its small size. While this facilitates some studies and keeps rearing costs low, any research that relies on tissue sampling is somewhat hindered. For example, analyses that require large volumes of blood or enzymatic assays that require large amounts of tissue are challenging in the zebrafish. In these cases, other fish models may be more appropriate (Hill et al. 2005).

The zebrafish is also currently being used to study the genomic effects of sex hormones. For example, Santos et al. (2007) studied the effect of estradiol on the transcriptome of the zebrafish, finding large-scale changes that in some cases were consistent with genes that are known to be sexually dimorphic in the zebrafish liver (Robison et al. 2008). Other studies of sexual dimorphism in the transcriptomes of other tissues are now emerging, including the brain (Santos et al. 2008) and reproductive tissues (Riggio et al. 2000; Menuet et al. 2002).

The degree to which toxicological data from zebrafish can be translated to aquaculture species is currently unknown, and almost certainly depends on the nature of the perturbation and the genetic and physiological mechanisms that regulate the response.

Nutrition

There has been some recent interest in using the zebrafish as a model for fish nutrition. For example, Robison et al. (2008) showed that manipulation of dietary carbohydrate had significant impacts on the hepatic transcriptome and that many of these effects were sex specific. The potential for sexually dimorphic responses to nutritional

manipulations has not been fully investigated in aquaculture species. This is an example of a model species generating testable hypotheses for aquaculture species. The fact that the liver was highly sexually dimorphic in this study suggested that hepatic sexual dimorphism may be conserved with mammals (Amador-Noguez et al. 2005; Rinn and Snyder 2005). Other researchers have examined sexual dimorphism in gene expression in the zebrafish, but not in the context of a nutritional manipulation (Sreenivasan et al. 2008).

A number of metabolic pathways are being studied in great detail using teleost model species. For example, zebrafish are being used to study the metabolic and genetic basis of copper toxicity (Hernandez and Allende 2008), as a bioassay for screening genes that regulate obesity (Jones et al. 2008), and for vitamin metabolism and regulation (Ross and Zolfaghari 2004). In one study, cytochrome P450 was linked to nutrition and metabolic status in zebrafish from early on in development and postembryonically in regulating the metabolism of retinoic acid (Gu et al. 2006). Recently, a gene that is known to be involved in liver and glucose metabolism has been cloned, and treatment with the protein product was found to induce expression of genes involved in cholesterol and lipid pathways (Archer et al. 2008). For several genes involved in nutrient regulation, zebrafish was the species in which they were cloned and first studied. This information has facilitated their cloning and studies involving their activity in aquaculture fish species (Seiliez et al. 2001; Taylor et al. 2004). Comparisons of the desaturation and elongation of fatty acids between zebrafish and Nile tilapia have also been used to study the conversion and storage of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) omega-3 fatty acids. These studies found that fish with increased enzymatic activity were still unable to more efficiently convert oleic oils into EPA and DHA in fillet tissue to levels found when the fish were fed a diet containing fish oil (Tocher et al. 2001). Findings such as these could have major implications on the formulation of diets for commercial aquaculture species. Other studies in zebrafish have shown a correlation between faster growth rates and diets containing high levels of polyunsaturated fatty acids (Meinelt 2000).

Relative to how nutrient level regulates metabolic activity and growth, zebrafish were used to identify *rapunzel*, a nutrition-dependent factor for growth control. *rapunzel* is a fin overgrowth mutant, and studies found that even with poor nutrition this mutant would bypass normal growth checkpoints designed to limit energy output and growth under these conditions (Iovine and Johnson 2000). Other related studies include regulation of carbohydrate metabolism, growth factors, and glucose transporter genes in regard to carbohydrate utilization in diets (Duan 1998; Nemeth and Ganz 2006). While these studies probably have a relatively high correlation with finding in other cyprinids and omnivorous fish, carbohydrate utilization in piscivorous fish is very inefficient and research has yet been unable to precisely determine where this pathway is uncoupled relative to processing carbohydrates as energy sources (Hilton and Atkinson 1982; Pitkanen et al. 1999).

Changes in dietary nutrients also affect spawning and development in zebrafish (Markovich et al. 2007). Hormonal and cellular changes found in female zebrafish related to lipid uptake during egg development and prior to spawning are believed to be characteristic of changes that occur in other teleosts (Peute et al. 1978). Other work has used zebrafish as a model to understand the intestinal bacteria that reside in healthy fish and how relative levels and community composition of bacteria can change according to dietary changes (Rawls et al. 2003). Furthermore, gnotobiotic zebrafish have been created to understand the role of certain bacteria in intestinal

absorption of nutrients and to study host response to different types of bacteria (Rawls et al. 2004). Other related studies using zebrafish have demonstrated how changes in the intestinal flora can influence the ability of the animal in handling different toxic compounds (Kasokat et al. 2008).

The response to starvation is another nutritional manipulation that is of considerable interest in aquaculture species. Recently, Drew et al. (2008) carried out a microarray study of the response to starvation in zebrafish brain and liver. They found little response in the brain, while the liver dramatically changed several biological processes, including biosynthesis, metabolism, oxidative stress, and the unfolded protein response. Remarkably, the hepatic response to starvation in the zebrafish was more similar to that observed in trout (Salem et al. 2005, 2006) than carp (Wang et al. 2006), although carp and zebrafish are both cyprinids. These results highlight the fact that some processes may be highly variable in the teleost lineage, and a comprehensive understanding of the genetics of the process needs to be achieved before generalizations across species can be made.

Conclusions

With the increasing importance of aquaculture as a world protein source, increased funding has become available for both private and academic researchers. This has expanded our knowledge base regarding the physiology, nutrition, and genomics of cultured fish species. However, there are still areas where research goals in aquaculture can be facilitated by incorporation of laboratory teleost models. Currently, model teleost species have considerable advantages with respect to genomics infrastructure. These advantages are likely to be relatively transient as new genomic technologies become more widely implemented. However, model teleosts will always harbor some logistical advantages, such as fast generation times and more forgiving space and rearing requirements. As the logistical advantages of model organisms will never go away, they will likely remain a useful means of answering questions of relevance to the aquaculture industry. Translating the results of these experiments to cultured species will critically depend on a deeper understanding of the patterns and processes of teleost evolution.

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Chapter 8
Clonal Lines and Chromosome Manipulation for Aquaculture Research and Production

Krista M. Nichols

Chromosome set manipulation in many aquaculture species has been used as a tool for monosex production, production of polyploids to induce sterility or to improve growth, and production of clonal lines for applied and basic research in quantitative trait analysis, genome architecture, and evolution. Several previous studies have extensively reviewed the utility and status of chromosome set manipulation in aquaculture (Tave 1993; Pandian and Koteeswaran 1998; Arai 2001; Felip et al. 2001; Hulata 2001), and more recently, the use of these techniques for the development of clonal lines in fishes (Komen and Thorgaard 2007). In this chapter, an overview is provided for the basic methodologies used for chromosome set manipulation in fish and shellfish aquaculture species, with special attention paid to recent advances in the use of these techniques for commercial aquaculture production, or improvement of strains used in aquaculture. Finally, the utilization of clonal lines for linkage mapping, quantitative trait analysis, and aquaculture production is reviewed, with a forward perspective on the use of clonal lines and chromosome set manipulation for future applications in aquaculture.

Chromosome Set Manipulation

With external fertilization in many aquaculture species, including fish and invertebrates, gametes are generally easily handled for chromosome set manipulation for the purposes of inducing (1) polyploidy and (2) all-paternal (androgenesis) or all-maternal (gynogenesis) inheritance. Methods for chromosome set manipulation have been reviewed extensively for aquaculture species (Thorgaard 1983; Tave 1990, 1993; Pandian and Koteeswaran 1998). Chromosome set manipulation generally includes the use of ultraviolet or ionizing radiation to preferentially inactivate or destroy nuclear genetic material in maternal or paternal gametes before fertilization (in the case of andro- or gynogenesis, respectively), and/or the application of chemical, pressure, or temperature shocks during meiosis or mitosis following fertilization to modify ploidy levels (for induced polyploidy, androgenesis, and gynogenesis; Figure 8.1).

Induced Polyploidy

Generally speaking, production of triploids and tetraploids is the most commonly used methods of chromosome set manipulation in aquaculture species. Both triploids and tetraploids are generated by shock treatment of eggs just after fertilization and

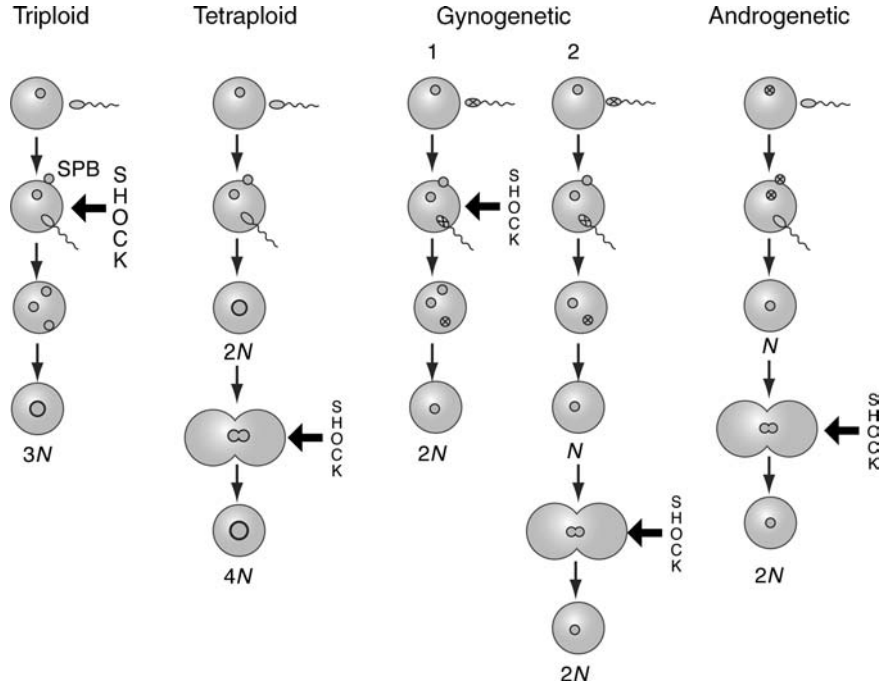

Figure 8.1. Schematic overview of gamete and embryo treatments for chromosome set manipulation. Techniques for production of triploid, tetraploid, gynogenetic, and androgenetic progeny are described fully in the text. Briefly, triploidy is accomplished with the retention of the second polar body during meiosis II using a shock treatment. Tetraploidy results from shock treatment that prevents the first cleavage that occurs after the first mitotic replication of chromosomes. Both andro- and gynogenesis are products of inactivation of either the maternal or paternal genome (denoted by "X" in respective gamete), respectively, and shock treatments either to retain the second polar body (gynogenesis 1) or to prevent the first cell cleavage after the first mitotic replication of chromosomes (gynogenesis 1 and androgenesis). *Source*: Tave (1990, 1993).

activation of embryogenesis by viable normal sperm (Thorgaard 1983; Tave 1993). Triploids are produced by inducing the retention of the second polar body in the egg after fertilization (Figure 8.1). The shock treatment applied to induce the retention of the second polar body includes temperature shock (heat or cold), hydrostatic pressure shock, or chemical shock treatment, with temperature shock being the most easily and commonly used method. The timing of treatment is dependent on the rate of development, which in turn can be influenced by the temperature at which fertilization and development occur; the timing and magnitude of treatment are also dependent on species, and in some cases intraspecific genetic background, owing to intraspecific differences in development rate (Streisinger et al. 1981). Tetraploids are produced by similar shock treatments that prevent the first cleavage of embryonic cells postfertilization after the first mitotic replication of chromosomes (Figure 8.1). Treatments used for induction of polyploidy in a variety of fish species are reviewed by Pandian and Koteeswaran (1998).

The success of induced triploidy or tetraploidy is varied, and depends heavily on optimizing the timing, duration, and magnitude of shock treatments. Using shock

treatments to induce triploidy and tetraploidy is much less successful than natural fertilization of 2*n* gametes produced by tetraploid individuals with 1*n* gametes from normal diploid individuals (Arai 2001). Success of induced polyploidy is evaluated with a variety of techniques (reviewed in Thorgaard et al. 1983; Tave 1993; Pandian and Koteeswaran 1998) including quantification of cell or nuclear volume, chromosome counting, determination of DNA content by flow cytometry (Allen 1983), and quantification of nucleoli. All of these techniques can be minimally invasive with the collection of blood from live animals. Evaluation of DNA content by flow cytometry is generally the most reliably and widely accepted method for detection of ploidy levels in aquaculture (Harrell et al. 1998).

Triploids and tetraploids are produced in aquaculture for several purposes including (1) production of sterile animals to minimize impacts of escaped or stocked organisms on natural populations and ecosystems (Beaumont 2000; Cotter et al. 2000; Kozfkay et al. 2006), (2) production of monosex stocks to eliminate sex-specific differences in maturity and growth in aquaculture, (3) potential improvement in aquaculture production traits such as growth rate, feed conversion efficiency, fillet quality, and disease resistance in the production of polyploids, and (4) production of tetraploid fish to facilitate the production of triploid progeny by cross-fertilization of gametes from tetraploid and normal diploid organisms (Arai 2001; Hulata 2001). The fish species for which triploids and tetraploids have been successfully produced, either experimentally or for aquaculture have been extensively reviewed elsewhere (Tave 1993; Arai 2001; Hulata 2001).

Induced polyploidy has been most successful for the aim of induced sterility and the production of monosex cultures (together with hormone treatment), but the idea that polyploid fish would be larger or faster growing has not consistently held true in some fish species studied (Ihssen et al. 1990; Tave 1993; Hussain et al. 1995; Dunham et al. 2000; Arai 2001; Blanc et al. 2001; Felip et al. 2001; Kerby et al. 2002). The general thought that triploids would show preferential partitioning of energy to growth rather than sexual maturation and gametogenesis, while normal diploids show partitioning of energy to reproduction rather than growth, has mixed and varied results in aquaculture fish species (see Dunham et al. 2000; Hulata 2001; Tiwary et al. 2004 for review). Some studies in salmonid fishes indicate that triploids, when compared to diploids, have equal or poorer performance in embryonic survival and juvenile and near market-size growth, length, and weight (Utter et al. 1983; Solar et al. 1984; Happe et al. 1988; Quillet and Gaignon 1990; McGeachy et al. 1995; Withler et al. 1995; Bonnet et al. 1999; O'Keefe and Benfey 1999; Sheehan et al. 1999; Johnson et al. 2004). On the other hand, some studies in salmonids have shown that triploids have greater or equal performance in growth rate relative to diploids (Oppedal et al. 2003). In one study, triploid rainbow trout (*Oncorhynchus mykiss*), although not showing improved growth rates, showed a greater carcass weight at market size compared to diploids, most likely due to the increase in food intake, rather than in food conversion efficiency (Muller-Belecke et al. 2006). In a related study in adult rainbow trout, triploids were shown to have improved fillet quality over diploid rainbow trout (Poontawee et al. 2007). In channel catfish (*Ictalurus punctatus*), triploids exhibit improved growth over diploids in experimental tank systems (Wolters et al. 1982), but in commercial rearing settings, triploids do not grow as fast as diploids (see Dunham et al. 2000 for review). In a few studies, other fish species have shown greater growth and performance in triploids relative to diploids. In turbot (*Scophthalmus maximus*), triploids and diploids showed similar growth from 6 to 24 months of age, but triploids older than 24 months (after

diploids began sexual maturation) were much larger than diploids (Cal et al. 2006). Chinese catfish (*Clarias fuscus*) triploids were larger in size and exhibited greater growth rates when reared at temperatures above 25°C (Qin et al. 1998).

Shellfish have more recently been used for induced polyploidy in aquaculture research, and show more consistent and promising results in growth and production qualities as polyploids relative to diploids, in contrast to what is found in fishes. Polyploidy has been successfully induced in a number of shellfish species, including the Pacific oyster (*Crassostrea gigas*; Guo et al. 1996), American oyster (*Crassostrea virginica*; Stanley et al. 1981), Japanese pearl oyster (*Nodipecten subnodosus*; Wada et al. 1989), Sydney rock oyster (*Saccostrea commercialis*; Nell et al. 1994), dwarf surfclam (*Mulinia lateralis*; Yang and Guo 2006), Pacific red abalone (*Haliotis rufescens*; Maldonado et al. 2001), greenlip abalone (*Haliotis laevigata*; Dunstan et al. 2007), Australian blacklip abalone (*Haliotis rubr*; Liu et al. 2004), Zhikong scallop (*Chlamys farreri*; Yang et al. 2000), bay scallop (*Argopecten irradians*; Tabarini 1984), lion paw scallop (*Nodipecten subnodosus*; Maldonado-Amparo et al. 2004), catarina scallop (*Argopecten ventricosus*; Ruiz-Verdugo et al. 2000), northern quahog (*Mercenaria mercenaria*; Eversole et al. 1996), Kuruma shrimp (*Marsupenaeus* (*Penaeus*) *japonicus*; Sellars et al. 2006a; Sellars et al. 2006b), Chinese shrimp (*Fenneropenaeus chinensis*; Li et al. 2003), and common mussels (*Mytilus edulis*; Beaumont and Kelly 1989). The preferred method for induction of polyploidy in most shellfish species listed has been by chemical shock treatment, primarily cytochalasin B. Triploid Pacific oysters have reached large-scale aquaculture production in Australia, Japan, and the USA (Hulata 2001; Nell 2002), and Catarina scallops are produced for aquaculture in Mexico (Hulata 2001), but no other induced polyploid shellfish species has been used for large-scale aquaculture production. Induction of polyploidy in shellfish, like in fish, causes decreased survivability during the earliest stages of development, probably due to the adverse effects of the shock treatment. In contrast to fishes, however, most polyploid shellfish have shown improved growth compared to diploid organisms. Triploid oysters exhibit increased growth rates relative to diploids (Nell et al. 1994; Guo et al. 1996; Nell and Perkins 2005; Mallia et al. 2006). The improvement in growth rates in Pacific oysters has been shown to be likely associated with allelic diversity within triploids (Hawkins et al. 2000), or due to the masking of deleterious or slightly deleterious alleles for growth in polyploid individuals (Zouros et al. 1996). In scallop species studied, triploids also show greater growth in some studies (Ruiz-Verdugo et al. 2000; Yang et al. 2000), and no improvement in others (Maldonado-Amparo et al. 2004). In Chinese shrimp, triploids exhibit better growth performance and larger size during the usual maturation stage relative to diploid individuals (Li et al. 2006; Xiang et al. 2006). The dwarf surfclam (Guo and Allen 1994), common mussel (Brake et al. 2004), and quahog (Eversole et al. 1996) all exhibit greater growth and size, particularly at grow-out ages, relative to diploids.

Induced Gynogenesis and Androgenesis

The successful production of individuals with all-paternal (androgenesis) or all-maternal (gynogenesis) inheritance has been realized only in the past few decades of aquaculture and fish research. Androgenesis and gynogenesis in aquaculture have primarily been utilized for gene–centromere mapping (Danzmann and Gharbi 2001),

and to a lesser extent for the production of clonal or isogenic lines in two generations (Komen and Thorgaard 2007). With andro- or gynogenesis, complete inbreeding within individuals can be accomplished in one generation, producing diploid individuals that are homozygous at every locus. In the case that andro- or gynogenesis is conducted in the first generation using outbred gametes, progeny produced, while homozygous at every locus, will each be genetically unique descendants of unique gametes produced by meiosis in the parent generation. Clonal, isogenic, or genetically identical lines of fish can then be produced from first-generation androgens or gynogens, as described below.

As with induced polyploidy, the production of diploid individuals with all paternal or all maternal inheritance is accomplished by similar treatments of gametes with pressure, temperature, or chemical shock after fertilization has occurred. However, in this case, the nuclear genetic material or DNA of one parent is inactivated or destroyed prior to fertilization (Figure 8.1). When inactivated eggs are fertilized with viable sperm, all nuclear genetic material is inherited from the father (androgenesis); when unmanipulated, viable eggs are fertilized with inactivated sperm, inherited nuclear DNA comes only from the mother (gynogenesis). To inactivate genetic material in the egg or sperm prior to fertilization, gamma irradiation (often in the form of ^{60}Co and ^{137}Cs), X-ray irradiation, or ultraviolet (UV) light are used (Thorgaard 1983). Gamma and X-ray irradiation function to break the chromosomes in exposed gametes and require more specialized equipment and training for treatment of gametes. UV light inactivates the DNA of exposed gametes by inducing the formation of thymine dimers, and relative to gamma or X-ray irradiation, is easy to use for inactivation of sperm. UV inactivation is most successful in gametes of small size, most notably sperm cells (Komen and Thorgaard 2007), as UV radiation is attenuated before it reaches the nucleus in cells of larger size; for the same reason, care must be taken in minimizing volume or mixing of gametes exposed to UV light, ensuring that all cells are exposed. With inactivation of the egg or sperm prior to fertilization, resultant embryos would possess a haploid set of chromosomes from the paternal or maternal gamete, respectively. To restore diploidy in these developing embryos, a heat or pressure shock is used either to induce the retention of the second polar body in the egg (meiotic gynogenesis) or to prevent the first mitotic cell cleavage from occurring (mitotic gyno- or androgenesis). Because mitotic andro- or gynogenesis double the haploid set of chromosomes in the sperm and eggs, individuals produced by this method are also sometimes called doubled haploids (Komen and Thorgaard 2007). For reviews of specific treatments used for gyno- or androgenesis for multiple fish species, see Pandian and Koteeswaran (1998) and Komen and Thorgaard (2007).

Gynogenetic diploid individuals can be produced in two ways following fertilization of eggs with inactivated sperm cells (Figure 8.1): (1) shock treatment to induce the retention of the second polar body, which is normally extruded during meiosis II after fertilization (meiotic gynogens) and (2) shock treatment to suppress the first cell division or cleavage following the first mitotic replication of chromosomes (mitotic gynogens) (Thorgaard 1983; Tave 1993). The production of meiotic or mitotic gynogens each has advantages and disadvantages for the utility and survivability of resulting embryos. In meiotic gynogens produced from outbred individuals, the union of the 1*n* egg cell nucleus with the 1*n* polar body normally extruded during the second stage of meiosis produces a 2*n* embryo that may be heterozygous at some loci in the genome. Although it has generally been postulated that meiotic gynogens would have

higher survivability than mitotic gynogens (produced from outbred eggs) due to the maintenance of heterozygous loci in some parts of the genome (described below under gene–centromere mapping) and masking of deleterious recessive alleles, recent studies have shown that meiotic gynogens do not necessarily have improved survival over mitotic gynogens (Kato et al. 2001; Patton et al. 2007). Meiotic gynogens can be produced when monosex female cultures are desired (in XX/XY female/male systems). However, it is more common (and more desirable) to use hormone sex reversal of XX females to phenotypic males to produce XX sperm for induced triploidy in normal XX females (producing XXX females), since both monosex and sterility may be desired to maximize growth potential in aquaculture (Tave 1993; Arai 2001; Hulata 2001). Meiotic gynogens are also useful in the mapping of genes or genetic loci relative to the centromere (gene–centromere mapping; see below for further discussion), since loci located further from the centromere are more likely to have undergone recombination. Mitotic gynogenesis aims to produce individuals that are homozygous at every locus (completely inbred). However, success of mitotic gynogenesis has been met with mixed results in fishes, as some attempts at mitotic gynogenesis have resulted in a mixed population of diploid individuals that are meiotic and mitotic gynogens (Young et al. 1996; Komen and Thorgaard 2007). Mechanisms for the unexpected production of meiotic gynogens during the intended production of mitotic gynogens are unknown, but many studies suggest that spontaneous retention of the second polar body occurs in some species of fish (Komen et al. 1991; Cherfas et al. 1995; Ezaz et al. 2004a). To confirm the success of mitotic gyno- or androgenesis, marker homozygosity is evaluated by DNA fingerprinting or single-locus analysis (Young et al. 1996). Because of the mixed success in producing diploid homozygous individuals by mitotic gynogenesis, androgenesis is preferred for first-generation production of completely inbred individuals from outbred gamete sources (Young et al. 1996). In fishes, both meiotic and mitotic gynogenesis are used thereafter in subsequent generations to produce isogenic lines from a single first-generation doubled haploid (Komen and Thorgaard 2007; discussed below).

Androgenesis or all-paternal inheritance is induced by the suppression of the first mitotic cleavage during embryonic development, after inactivated egg cells have been fertilized with viable sperm (Figure 8.1). Nuclear DNA in egg cells can be inactivated by the same methods as for sperm cells in gynogenesis, but gamma irradiation has been the most successful and the most widely used (Komen and Thorgaard 2007); UV light does not give consistent levels of inactivation for species with large or opaque eggs, as the light is attenuated at varying levels before it reaches the nucleus. For species in which it has been tested (only in fish species), andro- and gynogenesis have been equally successful in survivability and performance of fish (Komen and Thorgaard 2007).

The use of androgenesis in aquaculture is relatively new compared to gynogenesis, as it was long believed the important cytoplasmic constituents of the egg could be damaged during the inactivation process (Thorgaard 1983). Some studies have investigated the nature of damage to cytoplasmic components by gamma ray or UV inactivation. In salmonids, gamma irradiation of eggs did not induce point mutations in maternal mitochondrial DNA (May and Grewe 1993; Brown and Thorgaard 2002). Similarly, in Nile tilapia (*Oreochromis niloticus*), UV inactivation of maternal DNA did not apparently cause point mutations or damage to maternal mitochondrial DNA (Myers et al. 1995). At least one study (in common carp, *Cyprinus carpio*) has

hypothesized that UV or gamma irradiation can damage other maternal cytoplasmic components such as maternally derived RNAs or proteins, but this hypothesis is more difficult to examine (Bongers et al. 1995), and has not been fully explored.

Production of Clonal or Isogenic Lines

Clonal lines in fishes are produced in two generations by andro- or gynogenesis from gametes taken from source populations of interest. In the first generation, if androgenesis is used in species with an XX/XY (female/male) sex-determining system, resulting progeny will be XX females or YY males. To make clonal lines from these androgenetic XX or YY progeny, a second round of gyno- (for XX individuals) or androgenesis (for YY individuals) is performed using gametes from single individuals to make clonal or isogenic families. Once clonal lines have been initiated in this way, subsequent generations of these clonal lines can be propagated by andro- or gynogenesis. Due to the poor survival of progeny produced from andro- and gynogenesis in subsequent generations, which is likely due to shock treatment effects on developmental processes (Komen and Thorgaard 2007), traditional crossbreeding of males and females from the same clonal line may be preferred, but is not always easily practiced. In species with a presumed single-locus sex determination system (such as the XX/XY system), since clones will be either genotypic females or males, some individual clones must be sex-reversed using hormone treatments prior to sex differentiation (Devlin and Nagahama 2002), so that both eggs and sperm are available from the same isogenic line. In general, reversal of genetic males to phenotypic females has been difficult (Devlin and Nagahama 2002; Muller-Belecke and Horstgen-Schwark 2007). In practice, sex reversal of YY male clones to phenotypic females has been largely unsuccessful, but sex reversal of XX female clonal lines to phenotypic males has been possible in some species including Nile tilapia, rainbow trout, and common carp (Komen and Thorgaard 2007).

The production of clonal lines, as described above, results in a population of completely inbred ($F = 1$), genetically identical (isogenic) individuals that were descended from a single gamete in the first generation. Aquaculture species that have been successfully cloned and are routinely used in research include common carp (*C. carpio*; Komen et al. 1991; Bongers et al. 1998; Ben-Dom et al. 2001; Gomelsky 2003), Nile tilapia (*O. niloticus*; Hussain et al. 1998; Sarder et al. 1999; Muller-Belecke and Horstgen-Schwark 2000), amago salmon (*O. rhodorus*; Kobayashi et al. 1994), ayu (*Plecoglossus altivelis*; Han et al. 1991; Takagi et al. 1995), rainbow trout (*O. mykiss*; Scheerer et al. 1991; Quillet 1994; Young et al. 1996; Quillet et al. 2007), hirame or Japanese flounder (*Paralichthys olivaceus*; Hara et al. 1993; Yamamoto 1999), and red sea bream (*Pagrus major*; Kato et al. 2002). Isogenicity within clonal lines is generally confirmed using DNA fingerprinting (Han et al. 1992; Hara et al. 1993; Takagi et al. 1995; Young et al. 1996; Jenneckens et al. 1999; Muller-Belecke and Horstgen-Schwark 2000; Ben-Dom et al. 2001; Kato et al. 2002; Ezaz et al. 2004b); some researchers have also examined the acceptance of skin or tissue grafts in clonal or isogenic fish (Komen et al. 1991; Qin et al. 2002). It should be noted that gynogenetic clones are produced naturally in some finfish species important for aquaculture. These include the gibuna or silver crucian carp (*Carassius langsdorfii*; Dong et al. 1996; Muller-Belecke et al. 2002; Ohara et al. 2003), gibel carp (*C. gibelio*; Paschos

et al. 2004), and the loach (*Misgurnus anguillicaudatus*; Itono et al. 2007). To date, no shellfish species have been cloned. Production of completely inbred bivalve mollusks is not possible or advantageous due to the very high degree of genetic load observed across this major group of organisms including oysters (Launey and Hedgecock 2001). Inbred lines by traditional crossbreeding have been formed in Pacific oysters to study the degree of genetic load, overdominance, and heterosis within this species (Launey and Hedgecock 2001; Hedgecock et al. 2007).

In finfishes, although andro- and gynogenesis have been shown to produce similar yields in species in which it has been studied (Komen and Thorgaard 2007), androgenesis has been more widely used and successful in the production and propagation of clonal lines. The reason for this includes a faster time to sexual maturity in males, as well as the fact that fertility in andro- or gyno-genetic females is often significantly reduced. In many fish species in which clones or doubled haploids have been produced, females exhibit impaired fertility relative to males (Arai 2001; Komen and Thorgaard 2007). Moreover, normal diploid females generally take longer to reach sexual maturity compared to normal diploid males, and the same is true in androgenetic and gynogenetic individuals. Female progeny produced by andro- or gynogenesis tends to also have decreased egg viability, decreased ovulation rates, and reduced egg size in some species (Arai 2001; Komen and Thorgaard 2007).

In aquaculture fish species, clonal lines have primarily been explored or developed as a tool for genetic studies on size, growth, and sexual differentiation and maturation. In the published literature, it is clear that very few species have advanced beyond successful production of clonal lines in aquaculture fish species. Clonal lines have been produced in these species for genetic research, and in some cases to capitalize on potential heterosis produced by crosses made between lines (Komen et al. 1991; Yamamoto 1999; Arai 2001). In many cases, even when only one clonal line is used in genetic crosses, the background of that clonal line contributes that same information to each individual produced as a result of the progeny, reducing some of the background genetic noise for studies of phenotypic traits. In common carp, clonal lines have been used to investigate spermatogenesis, sex differentiation, timing of sexual maturation, and genetic analysis of genome regions associated with stress response (Komen and Thorgaard 2007). In rainbow trout, crosses between clonal lines have largely been used for the development of genetic linkage maps (Young et al. 1998; Thorgaard et al. 2002; Nichols et al. 2003b) and in exploring the genomic loci and effects of loci with phenotypic traits that are divergent between clonal lines, a method known as quantitative trait loci (QTL) analyses (described below). In tilapia, clonal lines have been used to understand the role of environment on physiological and developmental differences (Muller-Belecke 2005). These investigators have further characterized growth performance in a number of clonal lines of tilapia and have produced a YY male clonal line with exceptional growth for potential aquaculture production, since male tilapia show better growth than females (Muller-Belecke and Horstgen-Schwark 2007). In amago salmon, androgenetic YY male fish have been used to investigate the effects of xenoestrogens on gonadal differentiation in XY males produced by crossing YY androgens with XX female amago salmon (Nakamura et al. 2002). Clonal lines of ayu have been used to investigate the heritability of body size, morphology, and meristic traits (Taniguchi et al. 1996). Clonal lines in rainbow trout show additional promise in evaluating the importance of cytonuclear interaction on phenotypic trait expression. Using different outbred egg sources to produce multiple families of the

same clonal line in rainbow trout, interaction of variable mitochondrial haplotypes with the same nuclear genome can be evaluated (Brown et al. 2006); in these studies, the authors found that variation in mitochondria diversity in the same nuclear background was significantly associated with oxygen consumption and development rate.

Gamete Cryopreservation

In aquaculture species such as rainbow trout, Pacific salmon (*Oncorhynchus* spp.), common carp, and oysters, male gametes are routinely stored under liquid nitrogen (cryopreservation) for later use (Hulata 2001). Cryopreservation has been generally used in finfish as insurance or preservation of gametes of imperiled or threatened natural populations, with the idea that future generations of these species may be resurrected from these banked stores using chromosome set technologies (Hulata 2001; Komen and Thorgaard 2007). In aquaculture fish species, male gametes are preserved as insurance in the case that improved lines or stocks are unintentionally eradicated by disease or some other unknown catastrophe and to insure that male gametes are available at all times for crossing when desirable females are sexually mature, and have been utilized in androgenesis. Although the conditions for freezing and cryoprotectant solutions have been determined for male fish gametes, the preservation of female gametes for long-term storage has not been successful. Cryopreservation of embryos and larvae has been possible in mollusk species (Hulata 2001).

Clonal Lines for Research in Aquaculture

Linkage and Gene–Centromere Mapping

Of the numerous genetic linkage maps existing now for aquaculture species (Danzmann and Gharbi 2007), several maps have used meiotic gynogens for the mapping of genes or markers relative to the centromere (gene–centromere mapping), or have used clonal lines for linkage mapping. Among these, gene–centromere mapping, also called half-tetrad analysis, has been used as a method for determining the relative position of markers or genes to the centromere on each chromosome (Danzmann and Gharbi 2001). To produce mapping populations for gene–centromere mapping, shock treatments are used to retain the second polar body after fertilization (meiotic gynogenesis). The retention of the second polar body results in a developing embryo that has retained, for each chromosome, both copies of the sister chromatids that are produced during interphase of meiosis I. Sister chromatids would be identical in alleles at every locus, if not for recombination with homologous chromosomes during meiosis I. Since only one of the sister chromatids for each chromosome will have undergone recombination with the homologous chromosome in any given cell, chromosome segments that were involved in recombination will exhibit heterozygosity. When recombination occurs randomly between a particular locus or gene and the centromere, 66% of meiogynogenetic progeny are expected to be heterozygous at that locus (Thorgaard 1983). When genes are closer to the centromere, the number of progeny that are heterozygous is lower than this expectation, since recombination

is rarer adjacent to the centromere, relative to recombination observed along the chromosome arm or near the telomere. With crossover interference, heterozygosity at some loci exceeds this expectation. Gene–centromere mapping has been used in numerous aquaculture species, including rainbow trout (Sakamoto et al. 2000; O'Malley et al. 2003; Guyomard et al. 2006), brown trout (*Salmo trutta*; Gharbi et al. 2006), Japanese eel (*Anguilla japonica*; Nomura et al. 2006), tilapia (Ezaz et al. 2004b), Pacific oyster (Guo and Gaffney 1993; Li and Kijima 2006), Pacific abalone (Li and Kijima 2005), Atlantic halibut (*Hippoglossus hippoglossus*; Reid et al. 2007), channel catfish (Liu et al. 1992; Liu et al. 1996), and loach (Morishima et al. 2001). Gene–centromere mapping has most recently been used as a means to evaluate the relationships between linkage maps and physical chromosomes. Fluorescent in situ hybridization physical mapping of loci can be directly compared to the linkage map location of loci to evaluate linkage map coverage (Guyomard et al. 2006; Phillips et al. 2006).

Linkage maps using progeny from crosses between clonal lines are less commonly used than gene–centromere mapping in aquaculture species. Clonal line crosses to produce progeny for linkage mapping include the production of backcrosses, F_2 intercrosses, or recombinant doubled haploids (rDH). In aquaculture species, the only species for which clonal line crosses have been used for linkage mapping is rainbow trout (Young et al. 1998; Robison et al. 2001; Thorgaard et al. 2002; Nichols et al. 2003b; Zimmerman et al. 2004; Guyomard et al. 2006). Linkage maps constructed from line crosses in rainbow trout are products of observed recombination rates between the paternal and maternal genomes in the F_1 generation. In the case of rainbow trout mapping families produced at Washington State University (Young et al. 1998; Robison et al. 2001; Thorgaard et al. 2002; Nichols et al. 2003b; Zimmerman et al. 2004), a single female clonal line (XX) has been crossed with different male clonal lines (YY) to produce all male (XY) progeny in the F_1 generation. For rainbow trout mapping families produced by clonal line crosses at Institut National de la Recherche Agronomique (INRA; Guyomard et al. 2006), mitotic female doubled haploids (XX) were intercrossed by sex reversing some XX doubled haploids to phenotypic males, producing all female (XX) F_1 individuals. Recombination among genetic loci during meiosis in F_1 individuals forms the basis for computing genetic linkage among markers. In rainbow trout clonal line crosses, sperm or eggs from the F_1 clonal hybrid are used to make doubled haploids by andro- or gynogenesis, respectively. In salmonids, a high degree of crossover interference is observed (Thorgaard et al. 1983), whereby double crossovers in a single chromosome arm are extremely rare (Guyomard et al. 2006). Furthermore, recombination rates in males are greatly reduced relative to females in the salmonids; in fact, map distances calculated from recombination events occurring during meiosis in females tend to be much greater than map distances calculated from recombinant events that occur during male meiosis where direct map comparisons can be made (Table 8.1; Sakamoto et al. 2000; Danzmann et al. 2005).

Linkage mapping in progeny from crosses between clonal lines or andro- or gynogenetic doubled haploids has a number of advantages and disadvantages relative to outcross mapping panels, and most of this evidence comes from rainbow trout wherein mapping families are made from both cross types. In all cases in which line crosses have been utilized for genetic linkage mapping, rDH have been used (Young et al. 1998; Robison et al. 2001; Nichols et al. 2003b; Zimmerman et al. 2004; Guyomard et al. 2006). rDH are advantageous for several reasons: (1) since all individuals are

Table 8.1. Features of genetic linkage maps in rainbow trout mapping families produced from outcrossing of outbred individuals and by crossing andro- or gynogenetic doubled haploids or clonal lines.

	n	Cross type	Mapping panel	M or F map?	Map length (cM)	Number of markers	Reference
Outcrossed mapping families							
University of Guelph	48	BC	Lot 25	Both	M: 994 F: 2,330	1,439	Danzmann et al. (2005)
	86	BC	Lot 44	Both	M: 1214 F: 2,222	1,439	Danzmann et al. (2005)
Clonal or doubled haploid crosses							
INRA	120	rDH		F	2,750	902	Guyomard et al. (2006)
Washington State University	76	rDH	OSU × Arl	M	4,590	1,359	Nichols et al. (2003b)
	99	rDH	OSU × CW (meristic)	M	1,233	221	Nichols et al. (2004)
	50	rDH	OSU × CW (*Ceratomyxa shasta*)	M	934.1	313	Nichols et al. (2003a)
	554	rDH	OSU × CW (development rate)	M	1,332.5	172	Nichols et al. (2007)
	106	rDH	OSU × HC	M	2,872	369	Zimmerman et al. (2005)
	170	rDH	OSU × SW	M	1,265.2	201	Robison et al. (2001)

Mapping panel name is identified in the original reference, and refers to the family, lot, cross, or trait for which the mapping panel was produced.
BC, backcross; rDH, recombinant doubled haploids; M, male; F, female.

homozygous, dominant marker systems such as amplified fragment length polymorphic (AFLP) markers can be scored unambiguously and (2) rDH exhibit greater power in the detection of QTL relative to all other crossing designs (Martinez et al. 2002). The disadvantages of the use of clonal line crosses for genetic linkage mapping and further analyses include (1) the greater amount of time needed to establish clones or doubled haploids for crossing and (2) reduced survivability in andro- or gynogenesis relative to traditional cross-fertilization due to shock treatments. With traditional cross-fertilization from outbred source populations, the extra time needed to establish doubled haploids from outbred sperm in one extra generation or to establish clonal lines or families in two extra generations is avoided. However, the efficiency with which AFLP markers can be used, and the power in homogenizing the genetic background of individuals with the production of rDH is lost. Although survivability may be an issue with the shock treatments used for chromosome set manipulation, sample sizes used for mapping in outcrossed mapping panels have often been similar to those for recombinant doubled haploid mapping panels (Table 8.1). Furthermore, although not specific necessarily to the use of clonal or doubled haploid individuals for linkage map crosses, male maps may have reduced map resolution relative to maps based on meiosis occurring in females. In rainbow trout, male/female recombination rates have been reported to be as high as 4.31:1 on average for the whole genome, but recombination differences can vary between chromosomes and between chromosome segments (Danzmann et al. 2005). Existing rainbow trout maps illustrate the point that for most chromosomes, recombination in centromeric regions is greater in meioses occurring in females relative to males, while males tend to have greater recombination in the telomeric or distal portions of chromosomes (Sakamoto et al. 2000; Danzmann et al. 2005). However, whether male- or female-based maps, or outcrossed- or rDH-based maps provide better resolution for the order of markers and detection of QTL will largely depend on the genetic architecture of the trait, and the region of the genome to which markers or traits are mapped. These types of comparisons and studies have not been conducted in any other aquaculture species.

QTL Mapping

QTL mapping is an efficient means whereby a genetic linkage map, constructed from crosses divergent in phenotypic traits of interest, is scanned systematically for significant statistical associations between genotype and phenotype (Falconer and Mackay 1996; Doerge et al. 1997; Lynch and Walsh 1998). QTL mapping using progeny derived from chromosome set manipulation techniques has been conducted, to date, only in rainbow trout. In rainbow trout, rDH from clonal line crosses have identified significant QTL for embryonic development rate (Robison et al. 2001; Nichols et al. 2007), resistance to *Ceratomyxa shasta* (Nichols et al. 2003a), meristic elements (Nichols et al. 2004; Zimmerman et al. 2005), natural killer cell activity (Zimmerman et al. 2004), and morphological and physiological features of smoltification (Nichols et al. 2008). Simulation studies show that QTL analyses in doubled haploid designs have improved power over any other design in the detection of QTL on a genome-wide level (Martinez et al. 2002). rDH from a clonal line cross shows utility in detecting epistasis and genotype by environment interactions (Nichols et al. 2007). QTL mapping in both outcrossed and recombinant doubled haploid rainbow trout has identified

a number of important chromosomal regions associated with traits important for aquaculture, including growth and development rate, age at sexual maturity, and disease resistance (more detail on genome mapping can be found in Chapter 5). QTL mapping in all other aquaculture species is conducted primarily in outcrossed mapping panels.

Genetic and Genomic Resources

Genomic resources such as small- and large-insert libraries and the development of microarrays as tools for expression analyses have been discussed elsewhere (Chapters 4 and 5). However, the use of clonal, doubled haploid, or introgressed lines in these techniques and experiments deserves mentioning here. Several large-insert bacterial artificial chromosome (BAC) libraries have been constructed for aquaculture fish species (Clark 2003), and of those, two have been developed from rainbow trout clonal lines (Thorgaard et al. 2002). Benefits in using clonal lines for BAC library construction are most notable in fishes that are products of genome duplications, including the salmonids and cyprinids. Because duplicated genes have a high degree of sequence similarity, differentiation of duplicate genes from allelic variation at a single locus is not an issue in libraries produced from a completely inbred individual that is homozygous at every locus. This type of resource will facilitate large-scale genome sequencing and assembly. Large-scale microarrays (cDNA and oligos) have been developed for a number of aquaculture species (reviewed in Chapter 4), and some studies have used clonal lines for gene expression analyses (Bayne et al. 2006; Purcell et al. 2006). With a homogenous genetic background among biological replicates within lines or populations of interest, within-line variance is minimized relative to between-line variance; thus, improving the detection of genes differentially expressed between lines of interest.

Marker-Assisted Selection

Marker-assisted selection, once markers have been identified for significant associations with traits of commercial importance, has potential for the genetic improvement of aquaculture species (Sonesson 2007) As of yet, this technology has not been integrated into large-scale commercial aquaculture. In clonal line crosses of rainbow trout, marker-assisted selection has proven useful in the propagation of advanced backcross individuals, or individuals introgressed for a genome region of interest (Sundin et al. 2005). In this way, rainbow trout individuals (produced from a first-generation backcross between a fast and slow developing clonal line) with the genome region for fast development rate have been selected using markers linked to a major embryonic development rate QTL, for repeated backcrossing or introgression into a slow developing background. This type of analysis is useful in isolating the effects due to a specific locus in the genome after many generations of introgression, and can be combined with microarray analyses to specifically identify sets of genes differentially expressed as a result of variability in a specific region of the genome.

Clonal Lines for Aquaculture Production

To date, clonal lines have not been directly utilized for commercial aquaculture production. With long generation times in many aquaculture species, production of clonal lines in two generations, propagation of clonal lines, and performance and growth testing can take years to complete. Furthermore, reduction in survivability of andro- or gynogenetic clones produced by shock treatment will hinder mass production of clones relative to outcrossed individuals. Sex reversal of some individuals from a clonal line can be utilized to obtain gametes that can be used to propagate clonal lines by traditional crossbreeding, but female clonal lines routinely show reduced fertility compared to outbred individuals. One notable disadvantage of developing clonal lines for large-scale production is that isogenic lines, with complete inbreeding, will lack the genetic diversity to respond to selection. Although a clonal line or recombinant doubled haploid line may show better performance during the development and initiation stages in a given environment, any change in that environment (e.g., temperature, density, or disease agents), may have devastating consequences for the future of an isogenic line incapable of responding favorably to a change in environment.

Although clonal lines may not show great promise for direct use in large-scale commercial aquaculture production, clones have been and will continue to be utilized for research in aquaculture production traits. All male YY tilapia have been used to develop strains of all male XY tilapia with consistent growth and performance, and show promise for the production of a consistent commercial aquaculture product, but the YY source stock is not truly clonal or isogenic (Muller-Belecke and Horstgen-Schwark 2007). In hirame or Japanese flounder, prior research utilizing crosses between clonal lines illustrates the potential improvement in growth and production traits, but use of clonal hybrids has not yet been realized in this species (Arai 2001). QTL analyses in rainbow trout clonal lines will moreover continue to reveal the genetic architecture of complex traits, with particular power in dissecting the roles of epistasis and genotype by environment interactions in the expression of quantitative traits, and in revealing promising candidate genes underlying QTL with combined microarray expression and QTL analyses.

Future Research

It is clear that research utilizing clonal lines has contributed to the growing knowledge on genome organization and genetic architecture of commercially and evolutionarily important traits in some fish species, and that chromosome set manipulation techniques have been successful for the production of monosex and sterile populations for aquaculture. Tests on the viability and production of induced polyploids will continue to be an active area of research, particularly as new fish and shellfish species are developed for aquaculture production. Gene–centromere mapping in induced meiotic gynogens will continue to be an important tool in the development of genetic linkage maps in all aquaculture species, and in directly comparing linkage and physical maps. The utilization of clonal lines will continue to be an important tool for the genetic dissection of complex traits in fishes. Clonal line crosses and propagation of rDH show a promising future for the genetic dissection of particularly complex traits, where epistasis and genotype by environment interactions are important. Although

not studied in any aquaculture species to date, transgressive segregation in rDH may show particular promise in bringing together unique gene combinations for genetic improvement of aquaculture production traits.

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Chapter 9
Issues and Methodology for Development of Transgenic Fish for Aquaculture with a Focus on Growth Enhancement

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Introduction

The advent of gene transfer methods in model mammalian species in the early 1980s (Palmiter et al. 1982; Hammer et al. 1985a) provided impetus to genetically engineer a range of other vertebrates for applied purposes. In particular, the remarkable enhancement in growth rate of mice to transgene-derived overexpression of growth hormone (GH) led to extensive transgenic studies in mammals for terrestrial agricultural species (Pursel et al. 1989). For domesticated mammals, transgenesis resulted in only modest growth acceleration (compared to effects in mice) and some improvements in feed utilization, but were also associated with significant pleiotropic metabolic, physiological, and morphological abnormalities (Pursel et al. 1989; Rexroad et al. 1989; Pursel et al. 1997; Rozycki et al. 1999; Pursel et al. 2004; Adams and Briegel 2005). Thus, this technology has not been applied in commercial agriculture, although research to overcome some of these obstacles continues.

Parallel efforts to engineer fish began in the mid-1980s, including several aquacultured species (Hammer et al. 1985b; Zhu et al. 1985, 1986; Dunham et al. 1987; Fletcher et al. 1988; Guyomard et al. 1989; Penman et al. 1992). Early efforts were directed toward developing methods for gene transfer, but it was not until the early 1990s that the first phenotypic effect of transgenesis in a commercial species was observed using GH transgenes (see below) (Du et al. 1992; Dunham et al. 1992a). Initial focus was directed to development of enhanced strains for aquaculture (Fletcher and Davies 1991); however, to date, practical implementation of this technology has been elusive for regulatory, public perception, and scientific reasons. These issues, as well as basic methodology, traits being modified, phenotypic effects, and biocontainment approaches, are discussed below.

Methodology of Gene Transfer

Transformation of Gametes

Initial methodology for gene transfer in fish was modified microinjection techniques developed for amphibian and mammalian systems. Essentially, a fine needle (approximately 2-μm tip diameter) is inserted into newly fertilized eggs, and approximately

1–5 nL of linearized DNA solution is injected (Fletcher et al. 1988; Yoshizaki et al. 1989; Penman et al. 1990; Du et al. 1992; Devlin et al. 1995a). Excellent survival of injected embryos can be achieved (75–90%), particularly if hardening of the chorion is prevented to facilitate microinjection prior to egg activation (Ginsburg 1963; Fletcher et al. 1988; Yoshizaki et al. 1989; Devlin et al. 1995a). Transformation frequencies vary widely, from no or a few percent transgenic animals being recovered, to more than 50% (see Devlin 1997). However, assessing gene transfer success among studies is complicated as transformation is defined in some cases simply as gene transfer (i.e., into the recipient individual) versus generation of stable transformed lines in other cases. Nevertheless, generating transgenic fish can usually be readily achieved with reasonable effort from which multiple unique lines can be established and analyzed.

A major factor associated with microinjection methods is the random integration of different numbers of transgene constructs into different host chromosome sites. Further, integration into chromosomes often does not occur at the one-cell stage of development, resulting in a mosaic embryo containing the transgene in only a portion of cells, and hence less than Mendelian transmission frequencies from founders to F_1 progeny. Random transgene integration and variable structures can result in strong phenotypic variance among founder and F_1 lines (Devlin et al. 1995a; Rahman et al. 2000; Nam et al. 2001b; Devlin et al. 2004b). In most cases, variation in expression among lines is a very useful feature, since, currently, a gene construct can only be designed to alter phenotype with approximate precision using our present knowledge about promoter strength and physiological consequences. Thus, having a range of expression states allows selection of lines with appropriate phenotypic effects to meet experimental objectives. As more understanding of transgene functions emerge, as well as the consequences of specific genes, the incorporation of advanced features into transgenes (e.g., border elements) that buffer host chromosome position effects will be extremely useful (Caldovic et al. 1999).

Other methods for introducing transgenes into fish gametes have been explored. Electroporation of fertilized eggs (for species with small eggs) or sperm has been widely explored by several groups to generate founder transgenic fish in several model (zebrafish and medaka) and commercial (Chinook salmon, rainbow trout, tilapia, and catfish) species (Inoue et al. 1990; Muller et al. 1992, 1993; Ozato et al. 1992; Powers et al. 1992; Sin et al. 1993, 1994, 1997; Zhao et al. 1993; Murakami et al. 1994; Symonds et al. 1994; Sin 1995; Tsai et al. 1995a, 1995b, 1997; Chen and Tsay 1997; Chen et al. 2002; Venugopal et al. 1998; Sarmasik et al. 2002; Chun et al. 2006). Despite the breadth of effort in this area, gamete electroporation has not been as widely reported as microinjection to generate stable germ line transgenic strains of fish (Lu et al. 1992; Ono et al. 1997; Sheela et al. 1998; Sarmasik et al. 2002).

Lipid vesicle-based methods have also been shown to successfully transfer DNA to embryos in zebrafish (Robles and Cancela 2007), and to sea bream following testis injection (Lu et al. 2002). DNA/dendrimer mixtures applied to sperm have also facilitated gene transfer in loach (Yang et al. 2001b). In our own case with coho salmon, we have not been successful in using liposome-mediated approach to transfect sperm.

Pantropic viruses with a very broad host range have been successfully used for gene transfer in fish (Lin et al. 1994; Lu et al. 1997; Sarmasik et al. 2001). Despite the ease of DNA transfer using this approach, the application of virally mediated gene transfer could be associated with significant public perception issues if the objective is application in commercial aquaculture. Further research on the stability

of virally transmitted transgenes in the presence of endogenous host viral sequences will likely resolve uncertainty in this area.

Interestingly, it has been recently hypothesized that interspecies antifreeze gene transfer occurred in the wild via gamete-mediated transfer of exogenous DNA present in aquatic environments (Graham et al. 2008). Such data suggest that a natural mechanism for uptake of exogenous DNA exists, although a previous report indicated that direct treatment of gametes with DNA failed to generate transgenic fish (Chourrout and Perrot 1992).

Embryonic Stem Cells and Primordial Germ Cells

Targeting gametes (eggs or sperm) for gene transfer in fish has the convenience and simplicity associated with microinjection or electroporation methods, but is not highly conducive to sophisticated genetic transformation approaches available in mammalian systems. As indicated above, microinjection (in fish and mammals) generates transformed lines with uncontrolled integrated transgene characteristics. The use of embryonic stem (ES) cells has been pivotal in allowing the production of highly targeted genetic changes in mice, which allows very specific analysis of cellular and physiological pathways. The use of ES cells (Hong et al. 2004) or primordial germ cells (PGCs) in fish has similar potential to provide control of the genetic modifications being sought (e.g., knockouts, promoter replacements, and coding region alterations) through homologous recombination-mediated and site-directed mutagenesis. Cre-mediated recombination for homologous insertions has been demonstrated in zebrafish embryos (Liu et al. 2007) and in an ES cell line (Fan et al. 2006). This level of precision will revolutionize the field of fish transgenesis by allowing predictable genetic changes to be engineered. Thus, it is critical that ES cell systems be developed for aquaculture species to facilitate this technology.

Efforts are under way to develop ES cells for several species including zebrafish (Sun et al. 1995; Xing et al. 2008), medaka (Wakamatsu et al. 1994; Hong et al. 1998, 2004), seaperch (Chen et al. 2003), gilthead sea bream (Bejar et al. 2002), and the flatfish turbot (Holen and Hamre 2003). A critical requirement for application of ES cells to transgenesis is that they are able to be transferred into embryos and contribute to the normal growth and differentiation of a range of tissues. Transfer of ES or undifferentiated blastomere cells has been demonstrated for zebrafish (Ma et al. 2001), medaka (Wakamatsu et al. 1993; Hyodo and Matsuhashi 1994; Hong et al. 1998, 2004), and rainbow trout (Takeuchi et al. 2001). In one case, the cells had been previously genetically transformed with a reporter gene construct (Hong et al. 1998, 2004). PGCs have similar potential for transgenesis. Methods for mass purification of PGCs (from a strain containing a germ-line-specific promoter (vasa)/GFP transgene) have now been developed for rainbow trout (Takeuchi et al. 2002). These PGCs have been transferred successfully to embryos where they were incorporated into the germ line to yield functional gametes that yielded transgenic offspring (Takeuchi et al. 2003).

Structure of Integrated Transgenes

The structure of an integrated transgene plays a key role in the expression level and thus physiological consequences of the gene construct. Studies using Southern

blotting and in situ hybridization have been consistent with constructs organized in multiple concatemeric forms and physically inserted into chromosomes (Yoshizaki et al. 1991; Tewari et al. 1992; Dunham et al. 1992b; Devlin et al. 1995a; Devlin et al. 2004b). Partial analysis of transgene ends in carp (containing 200 construct copies) revealed multiple sites of integration and a range of DNA rearrangements (Wu et al. 2004, 2005). However, due to the complexity of transgenes within the strain examined, the exact structure of any one integration site could not be determined. Integration sites have also been studied in transgenic rohu using inverse polymerase chain reaction methods, and highly homologous sites have been identified in two cases suggesting that specific sites of integration may be preferred in this species (Rajesh and Majumdar 2006). Complete sequence analysis of an integrated copy of a GH construct in Atlantic salmon revealed that two rearranged copies of the original transgene had integrated into a 35-bp repeat region (Yaskowiak et al. 2006). The sequence of the integrated transgene did not differ from the injected construct, and the structure remained stable for at least four generations. For a different GH construct in coho salmon, four complete and two partial copies of the injected construct were found together organized in a direct tandem (head-to-tail) fashion (Uh et al. 2006). It was proposed that one mechanism for generating complex transgene integrants could arise from injected linear DNA molecules undergoing nonhomologous end joining to circularize the construct, followed by rolling-circle replication to create tandem repeats that are integrated into host chromosome breaks (Uh et al. 2006). This transgene was integrated into a complex area housing multiple repeat regions (some associated with horizontally transmitted DNA from a *Schistosoma*-like species), suggesting that hot-spots for insertion of foreign DNA may exist in the genome. Whatever the mechanisms used, it is clear from the few examples available that integrated transgenes can have complex structures that undoubtedly influence their function.

Promoter Sequences

Transgenic studies with fish were initially undertaken primarily with gene constructs derived from mammalian genome or viral sequences. Nonpiscine promoters have been shown to be effective in fish, in particular CMV-IE (e.g., Betancourt et al. 1993; Hwang et al. 2003; Martinez et al. 1996); however, the actin promoter from tilapia and carp has also been used to drive transgenes (e.g., Hwang et al. 2003). In general, it appears that fish promoters and coding regions express at higher levels and cause stronger phenotypic effects (Du et al. 1992; Devlin et al. 1994; Nam et al. 2001b, 2008) than do mammalian constructs. Table 9.1 illustrates the diversity of promoters that have been shown to be functional in cell transfection or transgenic studies within aquaculture species. With the advent of physical bacterial artificial chromosome maps and sequences for several model fish genomes (fugu, zebrafish, stickleback, and medaka), isolation of promoters has become greatly simplified. Similarly, expressed sequence tag information from a range of commercial species also facilitates isolation of piscine coding regions, obviating the need to use nonhomologous sequences in many cases (except where a novel function is being transferred) to modify production traits in aquaculture species.

Table 9.1. Promoters active (expression or phenotypic effects) in commercial species.

Promoter	Origin	Species	References
β-actin promoter	Carp, *Xenopus*, loach, sea bream, and tilapia	Loach, carp, trout, tilapia, rohu, sea bream, and green sunfish	Cao et al. (2005), Hinits and Moav (1999), Hwang et al. (2003), Kato et al. (2007), Liu et al. (1999, 2002), Moav et al. (1992), Morales et al. (2001), Nam et al. (1999, 2000, 2001b), Rosochacki et al. (1993), Venugopal et al. (2004), and Zhong et al. (2002))
5′ murine leukemia virus long terminal repeat	Mammalian virus	Bluegill and/or walleye check	Paul et al. (2001)
RSV-LTR	Chicken	Bluegill and/or walleye check, trout, tilapia, salmon, carp, and goldfish	Betancourt et al. (1993), Hallerman et al. (1990), Hernandez et al. (1993), Inoue et al. (1990), Lee et al. (2000), Paul et al. (2001), and Zhang et al. (1990)
CMV-IE	Cammalian virus	Bluegill and/or walleye check, trout, sea bream, tilapia, salmon, and catfish	Betancourt et al. (1993), Dunham et al. (2002c), Garcia-Pozo et al. (1998), Hernandez et al. (1993), Lee et al. (2000), Martinez et al. (1996), Paul et al. (2001), Takeuchi et al. (1999), and Tewari et al. (1992)
SV40	Mammalian virus	Trout	Inoue et al. (1990)
TK		Carp, trout, salmon, and tilapia	Betancourt et al. (1993) and Hernandez et al. (1993)
MMMV	Mammalian virus	Trout	Inoue et al. (1990)
JAK		Tilapia	Chen et al. (1997, 2000)
Vasa	Trout	Trout	Yoshizaki et al. (2000)
sGnRH	Atlantic salmon	Trout	Uzbekova et al. (2000a, 2000b)
MT-B	Sockeye salmon and mouse	Carp, trout, Arctic charr, Chinook salmon, and coho salmon	Chan and Devlin (1993), Devlin et al. (1994, 2004b), and Pitkänen et al. (1999)

(*continued*)

Table 9.1. (*Continued*)

Promoter	Origin	Species	References
m/hMT	Mouse and human	Atlantic salmon and loach	McEvoy et al. (1988) and Van et al. (2002)
H3	Sockeye salmon and Atlantic salmon	Carp, trout, Arctic charr, and Chinook salmon	Chan and Devlin (1993), Hanley et al. (1998), and Pitkänen et al. (1999)
EF1	*Xenopus*	Trout	Takeuchi et al. (1999)
Antifreeze	Ocean pout	Loach, Atlantic salmon, tilapia, trout, Chinook salmon, and cutthroat trout	Caelers et al. (2005), Devlin et al. (1995a), Du et al. (1992), Hobbs and Fletcher (2008), Rahman et al. (1998), Shears et al. (1991), and Tsai et al. (1995)
HSP70	*Drosophila*	Trout	Inoue et al. (1990)
GH	Trout, Chinook salmon	Trout	Bernardini et al. (1999) and Wong et al. (1996)
Prolactin	Atlantic or Chinook salmon	Trout	Amoros et al. (1998) and Uzbekova et al. (2003)

Traits Under Modification for Aquaculture

For aquaculture, traits being modified fall into two major groups: (1) enhancement of production efficiency to reduce costs and (2) modification of product characteristics to enhance value. In the former category, growth enhancement has been explored most extensively because of the historical endocrinological work that had demonstrated the responsiveness of fish to GH (Donaldson et al. 1979).

Growth Enhancement

Growth Hormone

Enhancement of growth rate has dominated transgenic research for commercial species because of correlated improvements in feed conversion efficiency. Primarily, GH genes have been used in several species (carps, catfish, and rainbow trout), initially with no, negative, or small (<20%) effects on body size and later with more dramatic results (Table 9.2). For example, a salmon GH cDNA transgene driven by an ocean pout antifreeze promoter resulted in a 3- to 6-fold increase in body size in Atlantic salmon (Du et al. 1992), and 10- to 30-fold increase in coho and Chinook salmon and rainbow trout (Devlin et al. 1995a). Similar dramatic effects (up to 37-fold) were also observed in coho salmon using a construct comprised of a full length sockeye salmon GH gene containing a promoter substituted from the metallothionein-B (Devlin et al. 1994) or histone H3 gene. These gene constructs have also been shown to strongly growth stimulate Arctic charr (Pitkaenen et al. 1999). Mud loach are

Table 9.2. Growth enhancement in transgenic fish using fish GH gene constructs.

Species	Promoter	Coding region	Growth effects (relative to control)	References
Atlantic salmon	opAFP	Chinook cDNA	3–6	Du et al. (1992)
Atlantic salmon	Atlantic GH1	Atlantic *GH1* gene	None	Lorens et al. (1990)
Atlantic salmon	MT-B	Sockeye *GH1* gene	~5	Nam et al. (2008)
Coho salmon	opAFP	Chinook cDNA	10–30	Devlin et al. (1995a)
Coho salmon	MT-B	Sockeye *GH1* gene	11–37	Devlin et al. (1994, 2004b)
Coho salmon	H-3	Sockeye *GH1* gene	~6	Nam et al. (2008)
Chinook salmon	opAFP	Chinook cDNA	6.2	Devlin et al. (1995a)
Rainbow trout (wild strain)	opAFP	Chinook cDNA cDNA	3.2	Devlin et al. (1995a)
Rainbow trout (wild strain)	MT-B	Sockeye *GH1* gene	17.3	Devlin et al. (2001)
Rainbow trout (domestic strain)	MT-B	Sockeye *GH1* gene	None	Devlin et al. (2001)
Cutthroat trout	opAFP	Chinook cDNA	10	Devlin et al. (1995a)
Arctic charr	MT-B	Sockeye *GH1* gene	14	Pitkänen et al. (1999)
Arctic charr	H3	Sockeye *GH1* gene	14	Pitkänen et al. (1999)
Arctic charr	CMV	Sockeye *GH1* gene	14	Pitkänen et al. (1999)
Loach	opAFP	Chinook GH1 cDNA	2.5	Tsai et al. (1995)
Loach	Loach β-actin	Loach GH gene	Up to 35	Nam et al. (2001b) and Venugopal et al. (2004)
Rohu	CMV	Rohu GH cDNA	4	Venugopal et al. (2004)
Rohu	Carp β-actin	Rohu GH cDNA	4.8–5.8	Venugopal et al. (2004)
Carp	RSV	rtGH1 cDNA	1.21–1.4	Zhang et al. (1990)
	RSV	rtGH1 cDNA	0.73–1.4	Chen et al. (1993)
Catfish	RSV	Coho GH cDNA	1.26	Dunham et al. (1992a)
Tilapia	CMV	Tilapia GH cDNA	1.82	Martinez et al. (1996)
Tilapia	opAFP	Chinook GH1 cDNA	3	Rahman et al. (1998)
Zebrafish	RSV	Coho GH cDNA	1.7	Zhao et al. (1993)
Zebrafish	Carp β-actin	Tilapia GH gene	1.2	Morales et al. (2001)

Adapted from Devlin (1997)

very responsive to growth stimulation (Nam et al. 2001b), and fast-growing fish have also been developed (see Table 9.2) for tilapia (Martinez et al. 1996; Rahman et al. 1998) and rohu (Venugopal et al. 2004), whereas significant but more modest effects have been seen in catfish (Dunham et al. 1992a) and carp (Fu et al. 1998). Whether the difference in growth effect among species arises from the use of homologous GH genes with superior physiological protein function or from more efficient expression of the transgene or both has not yet been completely clarified (Nam et al. 2008). It appears that sequences derived from the exact same species are not required (i.e., to produce allotransgenic fish) as very potent effects arise when, for example, an ocean pout antifreeze promoter driving a Chinook salmon cDNA or minigene is introduced into Atlantic or Pacific salmon (Du et al. 1992; Devlin et al. 1995a). Similarly, a metallothionein-B promoter/GH gene from sockeye salmon introduced into the very closely related coho salmon (Devlin et al. 1994; Devlin et al. 2004b) and rainbow trout (Devlin et al. 2001) also has very strong stimulating effects. Indeed, coho salmon containing nonhomologous sequences (from the related sockeye salmon) have shown the greatest growth effect of any transgenic fish to date, up to 37-fold weight gain compared to controls after 1 year of growth (Devlin et al. 1994). Similar effects have been observed in loach (Nam et al. 2001b), where a completely homologous transgene construct resulted in growth effects as high as 35-fold. The response of different species to GH gene transfer depends on many factors, including construct structure, site of integration, and the physiological capacity of the species and strain to respond. Some species appear to inherently have a greater capacity to respond to growth stimulation than others. For example, slow growing cold-water species such as salmon and trout are stimulated very strongly, relative to carps and tilapia that are already growing very rapidly (Table 9.2).

Other Growth Factors

Insulin-like growth factor-I (IGF-I) is normally positively stimulated by GH and is strongly associated with growth regulation. Transgenic mice overexpressing IGF-I show modest growth stimulation (1.3-fold) (Mathews et al. 1988), whereas efforts to generate fast-growing strains of domestic animals containing IGF-I constructs have generally not been successful (e.g., Pursel et al. 2004). Similarly, in coho salmon, it has not been possible to generate IGF-I transgenic fish using promoter vectors that allow production of GH transgenic fish. While it is known that treatment of fish with IGF-I protein in low doses can stimulate growth (McCormick et al. 1992), it is likely that unless precise expression of this hormone is achieved, lethal metabolic imbalance may ensue (e.g., hypoglycemia, via interaction of unbound IGF-I with the insulin receptor). Further, transgenic medaka overexpressing the Ea4 domain of IGF-I have been shown to have significant vasculature and hematological difficulties (Chun et al. 2006).

Overexpression of myostatin, a cellular signal that negatively regulates muscle fiber development, has been shown to reduce muscle growth in transgenic mice (Reisz-Porszasz et al. 2003). Conversely, expression of myostatin prodomain (which interferes with normal signaling) increases muscle mass in mice (Yang et al. 2001a). In fish, RNAi suppression of myostatin mRNA has been shown to enhance muscle growth in zebrafish (Acosta et al. 2005), and overexpression of the myostatin prodomain has been shown to alter muscle growth in zebrafish (Xu et al. 2003). In the mammalian

studies, significant alteration of musculature conformation occurs from myostatin overexpression. If similar effects occurred in fish, the visual appeal of transgenic fish could be altered that could affect their market acceptability.

Enhanced Disease Resistance

Survival of animals in aquaculture is strongly influenced by exposure to pathogens, particularly in facilities that utilize nonsterile rearing water (e.g., surface freshwater or marine aquaculture). Thus, management of diseases is a top priority for producers and creates a focus on enhancing innate and acquired disease resistance in cultured strains. Although immunity can be stimulated by vaccination, antibiotics, antimicrobial peptides, and immunostimulants (e.g., Jia et al. 2000), genetics remains a major approach to enhance disease resistance via selective breeding (Gjedrem 2000; Fjalestad et al. 2003). Transgenic approaches have also been explored via the overexpression of several immune-related genes in fish, with promising results. For example, zebrafish overexpressing lysozyme showed less than half the normal mortality from exposure to two different bacterial pathogens (Yazawa et al. 2006), and for grass carp, transgenic individuals overexpressing lactoferrin displayed significant resistance to a viral pathogen (Zhong et al. 2002). Strikingly, medaka and catfish transgenic for antimicrobial peptide constructs can show very strong protection from specific bacterial pathogens (Dunham et al. 2002c; Sarmasik et al. 2002), in some cases 100%. These experiments have shown that there is enormous promise in the application of transgenesis for enhancing fish health.

DNA vaccination involves the genetic modification of somatic cells (usually muscle cells) with gene constructs designed to express antigenic proteins from pathogens (Tonheim et al. 2008). The cellular rather than humoral presentation of antigens has the potential to stimulate the immune system more effectively, and indeed this approach has been shown to provide very effective protection against IHN and VHS viral pathogens (Traxler et al. 1999; Lorenzen et al. 2001). Thus, DNA vaccination has the potential to provide significant benefits for aquaculture producers and the environment by limiting disease progression and pathogen spread. Although these treatments do not target the germ line and, hence, resulting fish are usually considered not to be transgenic, DNA introduced into muscle tissue has been shown to move widely through the body (Tonheim et al. 2008). DNA mobility has raised unconfirmed speculation, and hence concern that the foreign DNA could find its way to gonadal tissue and integrate into the germ line to generate transgenic gametes and genetically modified progeny. Many studies to date have also used the CMV-IE promoter which, being of viral origin, may cause some public perception issues associated with mammalian viral sequences being present in muscle tissue of fish destined for human consumption.

Carbohydrate Metabolism

Growth modification has very strong indirect pleiotropic effects on many metabolic systems (see below). However, targeted modifications of specific metabolic pathways using transgenesis have also been undertaken in fish. Many commercially important

fish species (particularly salmonids) have relatively poor capacity for carbohydrate utilization, which has been hypothesized to be due to a poor ability to mobilize glucose into cells. To overcome this metabolic limitation, rainbow trout have been engineered with transgenes designed to overexpress hexokinase (HK; needed for phosphorylation of glucose) and the glucose transporter (GluT; required for uptake of glucose into cells) (Pitkänen et al. 1999). While limited metabolic effects were detected, further work in this area could assist in reducing significant feed costs associated with high-protein and -lipid diets.

Vitamin Independency

To maximize health and growth of fish, artificial diets for aquaculture benefit from addition of nonenergy supplements (minerals, vitamins, and cofactors). Vitamin C (L-ascorbic acid) is normally added to diets in large amounts as it cannot be synthesized directly by most fish species. However, several species of fish (Moreau and Dabrowski 2005) as well as some mammals possess the enzyme L-gulono-gamma-lactone oxidase that allows synthesis of vitamin C. Two studies have transferred the gene for this enzyme into fish (Toyohara et al. 1996; Krasnov et al. 1998). Although expression of this enzyme was not detected in rainbow trout, expression was detected in medaka suggesting there is potential for this approach to reduce vitamin C dependency. Similar approaches can be envisaged for other compounds required to provide optimum nutrition for fish in culture conditions.

Lipid Metabolism

While the previous examples of genetic modification have been primarily targeted toward enhancing production efficiency, product quality is also a key factor influencing consumer preference and marketability. In developed countries, a public awareness exists regarding the importance of consumption of omega-3 fatty acids (particularly EPA and DHA) for brain development, immune function, and prevention of heart disease. Thus, levels of these compounds in cultured fish (particularly salmon) are an important value criterion used by consumers for selecting fish as food. DHA and EPA omega-3 fatty acids cannot be synthesized by salmon, and thus must be derived from dietary sources since the enzyme needed to convert the parent fatty acids in diets (principally α-linolenic acid) is absent. To overcome this limitation, the gene for D6-desaturase has been transferred into zebrafish as a model species to determine whether new omega-3 fats could be endogenously produced (Alimuddin et al. 2005). These authors found a 1.4- and 2.1-fold elevation of EPA and DHA, respectively, in addition to a reduction in α-linolenic acid. Application of this technology in commercial aquaculture species has the potential to enhance the product quality beyond that achievable by nutritional approaches, and to allow production of these important lipids without reliance on marine sources of fish oil currently incorporated into commercial aquaculture diets.

Flesh Characteristics

Another approach taken to modify product quality has been to modify the changes (tenderization) occurring in muscle tissue following harvesting. Toyohara et al. (2005) have expressed a tissue inhibitor (TIMP) of the Matrix Metallo-Proteinase in medaka and have found that the flesh retains its strength characteristics longer than for control animals. Application of this technology is under way for sea bream with potential application to improve the quality of flesh following postharvest storage and transport.

Pleiotropic Phenotypic Effects in GH Transgenic Fish

Although transgene constructs aimed for use in aquaculture species both are well defined and often have a single aim (e.g., to enhance growth), it has become apparent that transgenes also cause a number of other phenotypic effects. Such pleiotropy may influence the feasibility of using a specific transgenic fish in aquaculture, and may also influence the ability to accurately and reliably estimate environmental impact of escaped or released transgenic fish into natural ecosystems (see below).

Pleiotropic effects can arise from direct actions of the transgene product (e.g., GH) or from indirect effects resulting from the biological effects of the transgene product (e.g., effects of rapid growth), and these effects may also in turn be influenced by each other as well as external environmental conditions. The following section focuses on pleiotropic effects observed in GH transgenic fish extrapolated from the wealth of studies that have been performed with such fish, and because these strains have been more highly targeted for commercial aquaculture applications.

Endocrine and Gene Expression Changes

Effects of GH transgenic fish (growth rate and enhanced feed intake) arise from pleiotropic effects of GH on the endocrine physiology of the animal. However, relatively little has been studied regarding the associated hormonal and gene expression changes in transgenic fish (in contrast to the abundant research on the endocrine systems in nontransgenic and GH-injected fish). Many GH transgenic fish studies have tested various body tissues for the presence of the transgene and its expression, and have found that most GH transgenes are incorporated and expressed in almost all tissues for transgenic coho salmon (Mori and Devlin 1999; Raven et al. 2008), Atlantic salmon (Hobbs and Fletcher 2008), tilapia (Chen et al. 1997; Hernandez et al. 1997; Caelers et al. 2005), Arctic charr (Pitkaenen et al. 1999), mud loach (Nam et al. 2001a, 2001b), silver sea bream (Lu et al. 2002), rohu (Venugopal et al. 2004), and ayu (Cheng et al. 2002). Notable exceptions to ectopic GH expression were found in the pituitary gland, where transgene expression was absent for one construct (SsGH2 mRNA) in the transgenic Arctic charr (Pitkaenen et al. 1999) and for another construct (OPAF-PcsGH, expressing Chinook GH) in the pituitary of transgenic tilapia (Caelers et al. 2005). Furthermore, combined endogenous and transgene GH expression has been found to be reduced in the pituitary gland of transgenic coho (Mori and Devlin 1999) and GH transgenic tilapia (Caelers et al. 2005). Negative feedback regulation acting

on the pituitary gland by transgene GH expression is likely occurring in these fish (Mori and Devlin 1999).

The few studies on GH transgenic fish that have specifically measured circulating GH have found elevated plasma levels of this hormone (Du et al. 1992; Devlin et al. 1994, 2000; Rahman et al. 1998; Mori and Devlin 1999; Kang and Devlin 2004; Raven et al. 2008).

Growth effects from enhanced GH production are thought to arise mainly through increased production of IGF-I. A comprehensive study by Eppler et al. (2007) on IGF-I in transgenic tilapia found that although growth was greater in transgenic individuals, serum IGF-I was lower. IGF-I protein, IGF-I-containing cells, and IGF-I mRNA levels were higher in the transgenic liver; and in muscle, IGF-I mRNA levels were twice as high as controls. Despite this, liver and serum displayed high IGF-I binding (which would reduce free IGF-I to interact with its receptor), suggesting that IGF-I could be acting in an autocrine or paracrine manner to stimulate growth. In contrast, transgenic coho salmon (with the opAFPGHc construct) had only slightly altered plasma IGF-I levels that were not consistent across experiments (Devlin et al. 2000). A later study on another strain of transgenic coho salmon (with a different construct, OnMTGH1) found that both plasma IGF-I and liver IGF-I mRNA were consistently increased (Raven et al. 2008). Furthermore, IGF-I was found in extrahepatic sites (Raven et al. 2008) where it may be operating in an autocrine/paracrine manner similar to GH transgenic tilapia (Caelers et al. 2005; Eppler et al. 2007). GH is expected to act through GH receptors (GHR) to stimulate IGF-I, but interestingly, transgenic coho salmon liver membrane GHR levels were not related to plasma IGF-I despite an increase in liver GHR and IGF-I mRNA expression in transgenic fish (Raven et al. 2008).

Ration level has been shown to strongly influence plasma IGF-I levels in nontransgenic fish (Duan 1998; Pierce et al. 2001), revealing the strong control that energy intake has on growth regulation. In GH transgenic coho salmon, ration restriction also lowered circulating IGF-I and IGF-I mRNA levels (despite the presence of high GH), suggesting that feed intake alters IGF-I production by influencing the target tissue rather than through the GH/IGF-I axis (which has been overridden in GH transgenic salmon). Of importance to aquaculture, GH transgenic fish maintained on a control ration level retain their very highly enhanced feeding motivation despite normal levels of IGF-I production. These data suggest that appetite is not directly influenced by circulating IGF-I levels.

Thyroid hormones and GH are known to be closely linked in their abilities to influence the growth and metabolism of vertebrates. Triiodothyronine (T3), the active form of T4, has been found to be higher in GH transgenic coho salmon (Eales et al. 2004; Kang and Devlin 2004). A reduction in inner-ring deiodinase enzyme activity converting T4 to T3 has been observed, indicating that T3 degradation has been decreased rather than T4 to T3 conversion enhanced (Eales et al. 2004). Thyroxine (T4) levels in GH transgenic coho salmon with the opAFPGHc gene construct were found to be lower than in similar size controls (Devlin et al. 2000), but were 1.5-fold higher than control fish in transgenic salmon with the OnMTGH1 construct (Kang and Devlin 2004). Distinct from effects on IGF-I, T3 and T4 levels were not found to be affected by ration level (Eales et al. 2004), suggesting that effects of GH on thyroid hormones may arise independently from IGF-I action.

GH-releasing hormone mRNA levels did not change in the hypothalamus of transgenic coho salmon, whereas the orexigenic and GH-stimulating hormone,

neuropeptide Y, was slightly lower in transgenic fish despite an observed increase in feeding motivation (Raven et al. 2008). The anorexigenic hormone, cholecystokinin (CCK), did not change in the telencephalon and hypothalamus of transgenic fish, although some indication of a lower level than in controls was observed in the telencephalon during winter (Lõhmus et al. 2008; Raven et al. 2008). CCK did not fluctuate seasonally in the brain as is typical of wild genotype coho salmon fish but did in the gut, and CCK receptor blockage increased feeding more in control than transgenic fish (Lõhmus et al. 2008), but only in winter. These data show how GH transgenesis can disrupt normal endocrine control of appetite, which in the case of GH transgenic coho salmon has become seasonally unregulated, distinct from that seen in wild genotype salmon that show strong winter suppression of appetite and growth (Devlin et al. 1994).

GH is a high-level control hormone that influences many physiological pathways, and as such it is anticipated that many genes in multiple tissues would be affected by GH transgenesis. Utilization of subtractive hybridization and microarray techniques to examine mRNA levels in liver and muscle has identified a plethora of gene expression changes. For example, microarray studies of transgenic coho liver have detected changes in genes involved in iron homeostasis, mitochondrial function, carbohydrate metabolism (pentose phosphate pathway), cellular proliferation, immunity, and hemoglobin production (Rise et al. 2006). In GH transgenic amago salmon containing the same gene construct, hepatic gene expression changes in similar pathways, iron-metabolism and immunity, as well as in reproduction and growth, were detected (Mori et al. 2007). Within the muscle of transgenic coho, gene expression patterns were found to be altered, particularly for myosin light chain-2 mRNA (Hill et al. 2000) as well as for myostatin 2 mRNA (Roberts et al. 2004). Furthermore, changes in myostatin immunoreactive protein indicate alterations to protein processing (Roberts et al. 2004). Further discussion of gene expression changes in GH transgenic salmon can be found in Chapter 4.

Metabolism and Energetic Changes

GH is known to enhance metabolism in vertebrate species through the mobilization of lipids, carbohydrates, and amino acids, and by stimulating protein synthesis (Harvey et al. 1995). Thus, increases in protein deposition and muscle mass in transgenic fish may well be expected to alter the metabolism of muscle and supporting tissues. Metabolic rates observed during resting (basal), routine activity, and postprandial periods as measured by oxygen consumption have been shown to be affected by GH transgenesis. Routine and standard oxygen consumption rates have been found to be increased in GH transgenic Atlantic salmon (Stevens et al. 1998; Stevens and Sutterlin 1999; Cook et al. 2000b; Deitch et al. 2006), yet in the same strain, another study found that over a prolonged time period (between first-feeding and smolt stages), cumulative oxygen consumption was less than in nontransgenic fish that take longer to reach the smolt stage (Cook et al. 2000b). Under starvation conditions, protein, lipid, and energy were utilized faster in transgenic fish than in controls (Cook et al. 2000c), which would suggest that routine (but not necessarily basal) metabolic rates have been increased. GH transgenic tilapia (McKenzie et al. 2003) and Arctic charr (Pitkänen et al. 2000) also have increased oxygen consumption rates. In contrast,

routine oxygen consumption was reduced in transgenic coho salmon, although this was suggested to be the result of poorer acclimation (and more erratic avoidance behavior) by nontransgenic fish and not due to a shift in basal metabolism (which did not differ between strains) (Leggatt et al. 2003). Thus, at least for coho salmon, GH appears to increase the scope for oxygen consumption rather than obligatorily increasing metabolic rate.

Maximum oxygen consumption (as determined during critical swimming speed) varies in transgenic fish, altering metabolic scope on a specific basis. Transgenic Atlantic salmon have no increase in maximum oxygen resulting in a decreased metabolic scope (Deitch et al. 2006), while transgenic tilapia have increased maximum oxygen and standard oxygen consumption allowing for a similar aerobic scope to nontransgenic fish (McKenzie et al. 2003). Transgenic coho salmon also showed an increased maximum oxygen consumption at critical swimming speed when compared to ocean-ranched fish (Lee et al. 2003). Oxygen consumption rates during periods of food scarcity and starvation have been found to vary depending on the transgenic species. After 24-hour postfeeding, Atlantic salmon have an increased oxygen consumption rate (Cook et al. 2000b); and for transgenic carp, oxygen usage was increased over controls for the first 96 hours of starvation (Guan et al. 2008). Transgenic coho salmon have elevated oxygen uptake after feeding, but this reduces to control levels after 4 days of starvation (Leggatt et al. 2003).

Differences in the oxygen needs of transgenic fish appear to differentially affect their ability to tolerate hypoxic conditions depending on species and ontogenetic stage. Transgenic carp have similar survival to their nontransgenic counterparts in a low-oxygen environment, yet the time to death was longer than for nontransgenic fish (Dunham et al. 2002a). Transgenic tilapia can regulate oxygen equally well as nontransgenic fish during hypoxia, and transgenic Atlantic salmon lost equilibrium at the same low-oxygen concentrations as control fish (Stevens et al. 1998). In contrast, hypoxia during embryonic development resulted in increased mortality of transgenic coho salmon, indicating that increased growth at this stage (Devlin et al. 1995b, 2004b; Sundstrom et al. 2005) reduces hypoxia tolerance (Sundt-Hansen et al. 2007).

Swimming physiology has been shown to be affected by GH transgenesis in several fish species. Farrell et al. (1997) found that fast-growing GH transgenic coho salmon were inferior swimmers to controls for both age-matched and size-matched fish, whereas in Atlantic salmon, one strain of transgenic fish has been found to have the same (Stevens et al. 1998) or different (Deitch et al. 2006) critical swimming speeds. No effect on swimming performance was detected in GH transgenic tilapia (McKenzie et al. 2003), whereas transgenic carp (Li et al. 2007) were poorer swimmers than nontransgenic controls. Deitch et al. (2006) suggest that swimming performance is limited by oxygen uptake, but other features may also play important roles in the biomechanics and physiology of swimming performance. Indeed, transgenic Atlantic salmon have shorter and thinner erythrocytes that likely have a higher surface area to volume ratio, although hematocrit, blood hemoglobin, and mean cellular hemoglobin content were found to be similar to nontransgenic fish (Cogswell et al. 2001). Further, poststress, transgenic Atlantic salmon showed a 14% increase in blood hemoglobin content (Deitch et al. 2006). Skeletal muscle tissues of transgenic Arctic charr and coho salmon show increased hyperplasia and an increase in muscle fibers (Hill et al. 2000; Pitkänen et al. 2000, 2001), and changes in myostatin 2 mRNA in transgenic coho salmon (Roberts et al. 2004) and modifications to myosatellite cell proliferation,

myogenin, Myo D I, and II mRNA in red muscle in transgenic Atlantic salmon (Levesque et al. 2008) could all be expected to influence muscle structure and hence swimming performance. Gill surface area was increased in transgenic Atlantic salmon due to an increase in gill filament length (Stevens and Sutterlin 1999), although this was not corroborated in a subsequent study (Deitch et al. 2006). Transgenic and control coho salmon also have not been found to differ in gill surface area (Stevens and Devlin 2000a).

Metabolic enzymes play key roles in supporting growth and activity in fish, and thus it is not surprising that these systems show modification in GH transgenic fish. For example, within transgenic coho salmon muscle, elevated phosphofructokinase (PFK) and cytochrome oxidase (CO) enzyme levels suggest greater glycolytic and energy generating capacity (Hill et al. 2000). Transgenic coho salmon also show increases in mRNA levels for enzymes involved in carbohydrate and amino acid metabolism (Rise et al. 2006). Transgenic Atlantic salmon were also found to have higher aerobic enzymes (citrate synthase and cytochrome oxidase) in red muscle and heart (Deitch et al. 2006). Transgenic juvenile tilapia show an increase in enzymes involved in gluconeogenesis from alanine and aspartic acid in muscle (Martinez et al. 2000), and in liver, an increase in pyruvate kinase suggested glucose was being used for energy (Martinez et al. 2000). On the other hand, adult transgenic tilapia showed no differences in enzyme levels (Martinez et al. 2000). However, in general, GH transgenic fish show elevated capacity for carbohydrate use and energy production as seen in other vertebrates. In particular, the former feature could play a significant role in aquaculture due to the economic benefits of carbohydrates in the formulation of aquaculture feeds.

Stress Tolerance

Stress in fish plays a major role in survival in both aquaculture and natural environments (Barton 2002) through influences on immune function, flight-or-fight responses, and competitive feeding success. Jhingan et al. (2003) did not find differences in cortisol or glucose levels between transgenic and control coho salmon following a handling stress or heat shock, whereas for Atlantic salmon, transgenic animals generally showed elevated catecholamine levels as well as altered hematology (Deitch et al. 2006). At a cellular level, Jhingan et al. (2003) found no consistent differences between genotypes for heat shock–induced heat shock proteins (HSP) induction or recovery. However, the glutathione system was enhanced in transgenic fish, perhaps to compensate for elevated levels of oxidants generated during rapid growth (Leggatt et al. 2007).

Osmoregulation

A key feature associated with the culture of salmonids (and other species that transition between fresh- and saltwater) is their ability to osmoregulate. For GH transgenic salmonids, their very rapid growth rate allows them to achieve smolt status (ability to osmoregulate and survive in seawater following juvenile rearing in freshwater) much sooner than for nontransgenic salmon (Devlin et al. 1994; Saunders et al. 1998; Devlin et al. 2000). Furthermore, Atlantic salmon showed increased Na^+/K^+ ATPase levels

typical of smolts and were unresponsive to the smolt-modifying effects of temperature and photoperiod (Saunders et al. 1998). In GH tilapia, seawater transfer tests did not increase plasma sodium levels (Fuente et al. 1999) indicating these fish had an enhanced ability to withstand sea and brackish water conditions compared to non-transgenic fish. Overall, GH transgenesis appear to enhance the ability of strains to osmoregulate and tolerate marine culture conditions.

Nutrition Requirements

GH transgenesis in fish generally increases feed intake and efficiency of feed utilization, with concomitant effects on growth rate. Transgenic tilapia (Rahman et al. 1998, 2001; Martinez et al. 2000; Kobayashi et al. 2007), coho salmon (Raven et al. 2006; Oakes et al. 2007), Atlantic salmon (Cook et al. 2000a), mud loach (Nam et al. 2001b), and cyprinid-mud loach crosses (Nam et al. 2004) show increased feed intake and enhanced feed conversion efficiency. It should be noted that transgenic coho had been found to have a lower feed conversion efficiency in one study (Kang and Devlin 2004), and reduced feed intake and feeding motivation have been seen in transgenic tilapia (Guillen et al. 1999b; Martinez et al. 2000). Transgenic rohu, like tilapia, consume food at a lower rate but still grow faster than controls owing to a more efficient utilization of feed (Venugopal et al. 2004), which compensated for lower feed intake and resulted in elevated growth rates. Transgenic carp have increased feed intake when consuming diets of low-protein content, but increased energy conversion efficiency when consuming high-protein diets (Fu et al. 1998; Xie et al. 2001). Studies with transgenic coho salmon have also found enhanced ability to deposit protein and improved use of carbohydrates (Raven et al. 2006). These shifts in diet utilization highlight the importance of diet composition when examining nutrient utilization in transgenic fish. Increased feed conversion efficiency in GH transgenic fish may be due, in part, to increased intestinal surface area (arising both from the transgene and from increased feed intake) as found for coho (Stevens and Devlin 2000b; Stevens and Devlin 2005) and Atlantic salmon (Stevens et al. 1999). However, there appears to be a limit to which increased feed intake and digestibility can compensate for decreased dietary energy while still maintaining growth. Transgenic coho salmon can increase feed intake as energy of the diet decreases, but maximum growth cannot be maintained after a point in which physiological limits to food intake (such as gut distention) are likely reached (Raven et al. 2006). A new metabolic and nutritional set point for energy intake and increased growth of these fish exists.

Elevated feed intake is often accompanied by an increase in the efficiency of protein, lipid, and energy utilization, but results do differ between species. For example, transgenic coho salmon have an increased ability to incorporate dietary protein and energy (Raven et al. 2006; Oakes et al. 2007), and transgenic tilapia have increased protein and energy digestibility (Rahman et al. 2001). However, no differences in protein or energy digestibility were found in transgenic Atlantic salmon (Cook et al. 2000a). Similar to coho, transgenic carp have increased protein recovery (Fu et al. 1998), but another GH transgenic carp did not show changes in energy reserves compared to controls, even during starvation (Guan et al. 2008). Arctic charr expressing salmon GH (same constructs as used in coho salmon) showed metabolic changes such that protein use appeared to be reduced with a shift toward increased utilization of lipid and nonprotein energy sources (Krasnov et al. 1999; Pitkaenen et al.

1999). As a result, protein excretion was less than in nontransgenic charr (Krasnov et al. 1999), potentially reducing nitrogenous waste in aquaculture conditions. Similarly, transgenic tilapia have increased protein retention and thus produce only 69% the ammonia-nitrogen waste as control fish (Kobayashi et al. 2007). Overall, the efficiency of protein and energy use is increased by GH transgenesis, but the extent of this change in efficiency is species dependent, and must be evaluated with diets designed to maximize the metabolic changes in these fish.

Flesh Quality

Muscle proximate compositions are critical determinates of whether transgenic fish strains would be used in aquaculture in the future. Fully fed GH transgenic coho have been found to have higher whole-body protein and lipid than nontransgenic fish in one study (Raven et al. 2006), but when fed a different diet did not differ from control fish (Oakes et al. 2007). When food intake is matched to control fish (i.e., pair feeding), transgenic coho had lower whole-body protein and lipid (Raven et al. 2006) or did not differ from control (Oakes et al. 2007). For transgenic Atlantic salmon, fully fed fish have decreased whole-body levels of protein and lipid and increased whole-body moisture (Cook et al. 2000a). While transgenic carp have increased protein content and reduced body lipid and moisture (Chatakondi et al. 1995; Fu et al. 1998; Dunham et al. 2002b) that results in increased dressing percentage (Fu et al. 1998). Contrary to other transgenic fish, the muscle composition of Arctic charr was unaffected by transgenesis (Krasnov et al. 1999; Pitkänen et al. 2000). In general, these data reveal that when provided with all the food they desire, transgenic fish can eat to excess and cause enhanced deposition of lipid, whereas when they are limited in their food intake, the lipolytic actions of GH are manifest and levels of lipid deposition are reduced to below that seen in nontransgenic fish. However, it is evident that transgenic technologies may alter fish composition, but these changes are very much affected by the construct, species, and diet utilized and must be assessed on a case-by-case basis.

Some studies have examined specific changes in amino acid and specific fatty acid levels in transgenic fish, characteristics which may affect marketability. For example, transgenic tilapia muscle was found to have lower levels of cholesterol, free alanine, and aspartic acid (Martinez et al. 1999). Some changes to amino acid levels were found in one type of transgenic carp, but in general, amino acid ratios and fatty acid profiles are similar to that seen in nontransgenic fish (Chatakondi et al. 1995; Fu et al. 2000; Dunham et al. 2002b).

In general, GH transgenesis has resulted in strains with improved energy conversion efficiency, resulting in leaner fish with a higher protein content. It will be critical to design feeds for aquaculture that meet the specific metabolic and compositional needs of fast-growing transgenic fish. In some cases, the use of costly or unsustainable feed ingredients (marine lipids and protein) may be reduced and shifted to plant-based protein sources, resulting in potential savings for the aquaculture industry.

Disease

GH treatments have been shown to stimulate immune function in fish (Kajita et al. 1992; Calduch-Giner et al. 1995; Sakai et al. 1996), suggesting that GH transgenic

fish should be more resistant to disease challenges. In GH transgenic carp, several cellular immune parameters were found to be stimulated (although age rather than size matched fish were used as controls), suggesting that GH overexpression could facilitate disease resistance (Wang et al. 2006). However, in a study directly examining the resistance of GH transgenic coho salmon to a pathogen (*Vibrio anguillarum*) challenge, Jhingan et al. (2003) found that at the fry stage no difference was detected from controls, whereas at the smolt stage a severe reduction in disease resistance was found. Although no effects on serum lysozyme levels were detected, these immune impairments appeared to be primarily mediated through the nonspecific immune system, since previously challenged fish showed equal resistance between genotypes. Two microarray studies have also found that several immune-related genes show depressed expression in GH transgenic salmon (Rise et al. 2006; Mori et al. 2007). Thus, effects on immune function can differ among strains/species of GH transgenic fish. The transgenic carp examined are much more modestly growth stimulated relative to the coho salmon; and thus, it is possible that at low-GH overexpression, immune function is stimulated, whereas at high expression, significant pleiotropic effects can ensue and cause impairment of immune function.

Acromegaly and Other Morphological Effects

A well-known normal function of GH in vertebrates is to stimulate bone and cartilage growth, both directly mediated through IGF-I and the thyroid hormone system (Harvey et al. 1995). Deficiencies in GH (via mutation or by hypophysectomy) lead to stunted growth, whereas overexpression can lead to significant morphological abnormalities including acromegaly syndrome which is associated with excessive cartilage and bone growth. GH transgenic fish can show similar abnormalities. For example, coho salmon transgenic for the opAFPGHc gene construct can show severe acromegaly with overgrowth of cartilage particularly in the head and operculum (Devlin et al. 1995b). Other strains of GH transgenic salmon using the same or a different (OnMTGH1) construct can show mild or no acromegaly effects (Ostenfeld et al. 1998; Devlin et al. 2000, 2004b). The effects appear not to be restricted to transgenic fish since domesticated strains of rainbow trout (which are already growing very rapidly) can show acromegaly effects when injected with GH protein, or made transgenic for GH (Devlin et al. 2001), an effect not seen in slower growing wild strains. In GH transgenic tilapia (Rahman et al. 1998) and carp (Dunham et al. 2002b), where growth stimulation effects are modest, changes in head shape have also been noted, but these are less severe than the abnormalities seen in salmonids. Thus, there appears to be a correlation between growth stimulation and abnormalities (Figure 9.1), with significant growth stimulation being possible without obvious morphological disruptions. However, severe effects can be found upon further growth stimulation.

Behavioral Effects

Many behavioral effects of transgenic fish are anticipated to influence productivity under aquaculture conditions, including effects on feeding motivation and interindividual interactions (e.g., aggression). These and other behavioral effects (antipredator

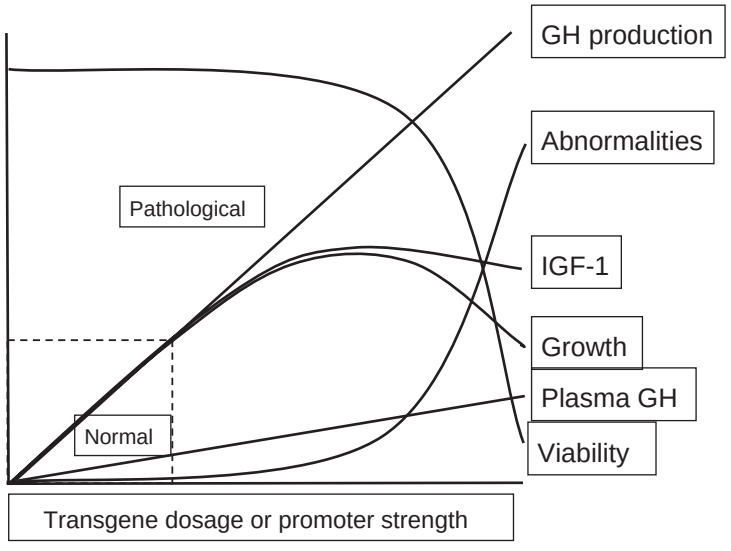

Figure 9.1. GH production and its effects on IGF-1, growth, and abnormalities in GH transgenic coho salmon.

responses, dispersal tendencies, and reproductive behavior) are critical for environmental risk assessments (see below) where they would be expected to influence the fitness and consequences of transgenic fish should they escape into natural environments.

Feeding Motivation

An anticipated effect of GH-transgenesis on behavior is an increase in appetite, and hence foraging behavior to provide enough food to support faster growth. Transgenic fish under hatchery conditions do indeed show a number of altered feeding behaviors, such as increased swimming activity and food intake (Abrahams and Sutterlin 1999; Devlin et al. 1999), remaining closer to the surface where food is normally delivered (Sundstrom et al. 2003), increased aggression during limited food conditions (Devlin et al. 2004c), and being less discriminate in trying novel food and inedible items (Sundstrom et al. 2004). Interestingly, some studies report that transgenic fish actually consume less food than wild-type fish (Martinez et al. 2000; Venugopal et al. 2004). Such effects may arise from rearing conditions prior to experiments (Guillen et al. 1999b; Sundstrom et al. 2007b) or may be a secondary effect of greater feed conversion efficiency still allowing for more rapid growth than in control fish (Venugopal et al. 2004). Some strains of transgenic fish have been shown to be able to increase food intake beyond their normally enhanced levels after being fed poor-quality feed (Fu et al. 1998; Raven et al. 2006), after a period of reduced feeding (Fu et al. 2007), or after hormone manipulation (Lõhmus et al. 2008), suggesting that they have an enhanced capacity for energy intake. At least for coho salmon, increased growth is achieved by maintaining a high-feeding behavior throughout the year, whereas wild-type fish reduce foraging behavior in the winter (Devlin et al. 1994, 2004b; Lõhmus et al. 2008). Clearly, enhanced appetites at all times of the year, coupled with improved

feed conversion efficiency and growth, are highly beneficial features for aquaculture production.

Under more natural conditions where fish were allowed to feed on live prey, transgenic fish grew better than wild-type fish, but the effects on growth were diminished relative to hatchery conditions with excess formulated feeds (Sundstrom et al. 2007b). In production trials in ponds (as opposed to research tanks), transgenic carp and tilapia were able to maintain greater growth than wild type (Rahman et al. 2001; Wu et al. 2003) when supplied with artificial feeds, revealing significant potential for these strains for aquaculture.

Antipredator Behavior

There is a close relationship between feeding and risk-taking behaviors (Lima and Dill 1990) since a hungry animal will typically be more willing to risk its life to meet its metabolic requirements. Not surprisingly, transgenic fish appear more willing to take risks to obtain food (Abrahams and Sutterlin 1999; Sundstrom et al. 2003). Indeed, in naturalized environments, transgenic salmon show reduced survival when reared with natural predators at early developmental stages (Sundstrom et al. 2004, 2005). The effects are stage- and species-specific; however, since no or variable differences in survival were found in the presence of predators for catfish and for salmon at the smolt stage (Dunham et al. 1999; Tymchuk et al. 2005). Because many fish predators are growth limited, achieving a large size rapidly may confer a reduction in predation risk, thus allowing fast-growing transgenic fish to outgrow predation sooner (Sundstrom et al. 2005), and possibly allow exploitation of novel resources to further enhance their growth advantage.

Aggression

Aggression between animals can result in dominance hierarchies within populations as well as cause stress effects. For GH transgenic fish, which are more highly motivated to obtain food than controls, aggressive behaviors have been noted. In a study with coho salmon, under low food availability, transgenic fish were found to be aggressive toward, and actually cannibalized, cohorts (Devlin et al. 2004c). In a feeding trial, pond-reared tilapia were more aggressive than transgenic fish that in turn were more aggressive than laboratory-reared nontransgenic conspecifics (Guillen et al. 1999b), showing the importance of rearing conditions on aggressive behavior (Sundström et al. 2003). However, under conditions of satiation feeding, transgenic coho salmon do not display any apparently greater aggression toward conspecifics than that observed for wild genotypes kept in tanks (personal observation).

Spawning

For some aquaculture species, natural breeding behavior is used to generate production animals, whereas for others, artificial spawning methods are used. In the former cases, and for risk assessments where reproductive fitness requires estimation, understanding a transgenic strain's reproductive capabilities is critical. For tilapia, GH transgenic males are able to breed with wild-type females that subsequently incubated the transgenic brood in their mouths, as is normal for the species (Abad et al. 2007).

Egg quality in transgenic carp was not different from wild type, and fertilization rate and hatchability were similar between the two genotypes (Wang et al. 2001). However, sexual maturity was reached later in transgenic male carps compared to wild type (Fu et al. 2005). In a study examining the reproductive success of transgenic coho salmon (Bessey et al. 2004), it was found that transgenic fish could naturally spawn and display breeding behavior, and were able to produce viable offspring under naturalized conditions. However, transgenic animals had very poor spawning success and showed reduced breeding behavior relative to wild-type fish from nature. Gamete quality was unaltered in males, but females produced smaller and more numerous eggs than wild fish. Transgenic animals also matured earlier than nontransgenic fish, a feature that could facilitate gene flow in populations. Caution was expressed, however (Bessey et al. 2004), since spawning success was strongly influenced by rearing conditions in that both wild type and transgenic fish reared under culture conditions throughout life were inferior spawners to wild-type fish recovered from nature (Bessey et al. 2004). Such genotype by environment (G×E) interactions make accurate assessments of complex traits of transgenic fish difficult to extrapolate to what would be found if these fish lived in the wild (Devlin et al. 2006).

Migration and Dispersal

While the mobility of transgenic fish is not a major issue in culture conditions, it is a critical feature that would influence the tendency of escaped animals to establish in novel habitats/areas. Changes in the animals' physiology may in some cases alter dispersal ability. For example, transgenic tilapia were found to osmoregulate better than nontransgenic and wild-type animals (Guillen et al. 1999a). If this affected their ability to survive and thrive in saltwater, the spread of transgenic tilapia between oceanic localities could be modified.

For other species such as salmonids, migration is a normal feature of their life history, moving at critical seasonal windows as smolts from freshwater river and lake systems into open ocean conditions, and subsequently returning to their natal freshwater rivers to spawn. Acquisition of the ability to osmoregulate in marine conditions, a process that is strongly size-related, is critical for successful smolt migration. For both growth-enhanced transgenic Atlantic (Saunders et al. 1998) and coho salmon (Devlin et al. 2000), the ability to osmoregulate and survive saltwater conditions is achieved at a younger age (but similar size) relative to controls. In transgenic Atlantic salmon, treatment with constant light and high temperatures, which separately can inhibit smolt development in wild-type fish, did not adversely affect transgenics. A critical question arises as to whether these fish would undertake seaward migration earlier, at the appropriate size, or wait to migrate until the right age and season but at a much larger size.

Dispersal tendencies acting on a small scale could also influence the fitness of GH transgenic fish in nature, by altering the ability to exploit new microhabitats with enhanced food resources or reduced predation risk or competition. In a study with coho salmon, it was found that GH transgenic fish had an enhanced exploratory tendency relative to wild type (Sundstrom et al. 2007a). The fish, as a population, also had a reduced tendency for cohesiveness which could influence their susceptibility to ambush attacks from predators.

Comparison of GH Transgenesis with Other Genetic Approaches (Domestication and Selection)

Aquaculture has utilized traditional genetic methods of selection and domestication for strain enhancement for many years (e.g., carps in Asia). Application of more sophisticated selective breeding programs for salmonids, carps, catfish, and tilapia (Fjalestad et al. 1993; Gjedrem 2000; Hulata 2001; Fjalestad et al. 2003; Dunham 2004) have resulted in remarkable improvements in growth (e.g., 7–10% per generation). Thus, currently, strains used in aquaculture are genetically selected for traits suitable for the culture environment, features which are distinct from traits found in wild strains. The remarkable enhancements in growth rates achieved by GH gene transgenesis (see above) have been demonstrated primarily in strains of wild origin that are also naturally slow growing, presumably as a consequence of naturally low-food levels as found in most temperate freshwater systems. From a commercial perspective, the potential of a transgenic strain must be compared not to wild type, but rather to the best domesticated strains currently available.

For rainbow trout, initial efforts to enhance growth occurred in domesticated strains using bovine GH gene constructs (a hormone known to potently stimulate growth in trout) were not successful (Guyomard et al. 1989; Penman et al. 1992). Subsequently, GH transgenesis was explored in trout using both slow-growing wild and fast-growing domesticated strains (Devlin et al. 1995a, 2001). Overexpression of GH in wild strains was found to have dramatic effects on growth as had been seen in previous studies with salmon, but the same transgene had no effect on growth in the fast-growing strain of domestic trout. The growth attained by the wild-strain transgenic fish was similar to that found in the nontransgenic and transgenic domestic strain, suggesting that the capacity for responding to GH had been diminished in the domesticated strain. In parallel, treatment of the wild and domesticated strains with a bovine GH formulation (Posilac^{TM}, Monsanto Corporation) revealed a much greater response in the wild strain (Devlin et al. 2001). Recently, the effects of Posilac have also been examined in wild and domesticated strains of Atlantic salmon confirming that a greater response is seen in the wild strains (Neregård et al. 2008). Thus, these data clearly show that the effect of GH (via transgenesis or direct treatment) are highly strain specific, and thus the lack of response seen in early GH transgenic trout trials may have arisen, in part, because these strains were already very fast growing. Some data exist, which suggest that circulating GH levels are elevated in domesticated Atlantic salmon; however, fish of radically different developmental stages were compared (Fleming et al. 2002), and downstream effects on IGF-I were not detected that is unusual. For coho salmon, both GH and IGF-I have been shown to be elevated in domesticated strains (Tymchuk submitted). Thus, domestication and GH transgenesis may be using, in part, similar cellular and physiological pathways to stimulate growth. Given these results, when undertaking transgenic experiments to enhance growth, preliminary studies to determine the responsiveness of a species and strain of interest to exogenous GH are advised. Similarly, when assessing the suitability of a transgenic strain for aquaculture, comparison to existing fast-growing strains will provide the preferable commercial comparison. However, for environmental risk assessments, comparing the phenotype of the transgenic strain to wild-type fish from nature is required.

Environmental Safety and Risk Assessment

A major factor influencing whether transgenic fish will be used in the future or not centers on environmental safety (Kapuscinski et al. 2008). From inception of this technology's development in fish, environmental risk assessment issues have been outlined and discussed at length (Tiedje et al. 1989; Hallerman and Kapuscinski 1990; Kapuscinski and Hallerman 1991; Devlin and Donaldson 1992). Major issues of concern surround the potential for transgenic fish to escape from the confines of aquaculture, and if so, whether they possess characteristics that would cause harm to ecosystem components that differ from those caused by wild-type fish (Devlin et al. 2006, 2007).

Many of the traits associated with transgenic fish discussed above would have clear influences on the fitness and consequence of transgenic fish in nature. For example, feeding motivation, disease resistance, spawning behavior, aggressiveness, dispersal and migration, and predator sensitivity are all expected to influence the ability of fish to survive and persist in nature. Similarly, competitive food acquisition is also expected to influence the impact transgenic fish would have on other ecosystem components (e.g., prey and conspecifics) with potential for hard-to-predict trophic cascades.

A significant challenge associated with environmental risk assessments is to generate data that are valid for natural scenarios. Laboratory-based experiments can mimic nature to a degree, but realistically for most species these experiments do not fully represent the natural environmental conditions that transgenic fish will encounter. Laboratory experiments are useful for identifying traits that differ between wild and transgenic strains, but the magnitude of the effects is probably different from those found if the fish were reared and studied entirely in the wild (Sundstrom et al. 2007b). However, relatively simple laboratory-based experiments can identify the major forces at work, and can form a framework for designing more complex experiments where nature is mimicked as much as possible.

G×E interactions also pose a significant problem for risk assessments. With the interplay between genes and environment shaping phenotypes (Schlichting and Pigliucci 1998), it is not surprising that transgenic and wild-type fish display different reaction norms: that is, they respond differently across a range of environments. G×E effects can arise from both the experimental conditions as well as the rearing conditions used to produce experimental animals. These interaction effects can even act on a population level (e.g., differential survival) influencing which animals may be available for study later on. For transgenic coho salmon, rearing animals with differing food availabilities (Devlin et al. 2004c) resulted in strong differences in survival of populations (i.e., populations containing transgenic fish under low food conditions crashed). Major effects of rearing conditions have been noted for both early (stream) and late (spawning) life history stages such that the relationship between transgenic and wild fish (growth and spawning ability) was found to differ depending on whether fish were reared in naturalized or culture conditions (Bessey et al. 2004; Sundstrom et al. 2007b). A strong G×E effect acting on growth (Figure 9.2) and predation behavior was observed in transgenic and wild-type coho salmon reared both in the hatchery and in a simulated natural stream (Sundstrom et al. 2007b). In this case, hatchery-reared transgenic fish were stronger predators in a simulated natural stream than corresponding

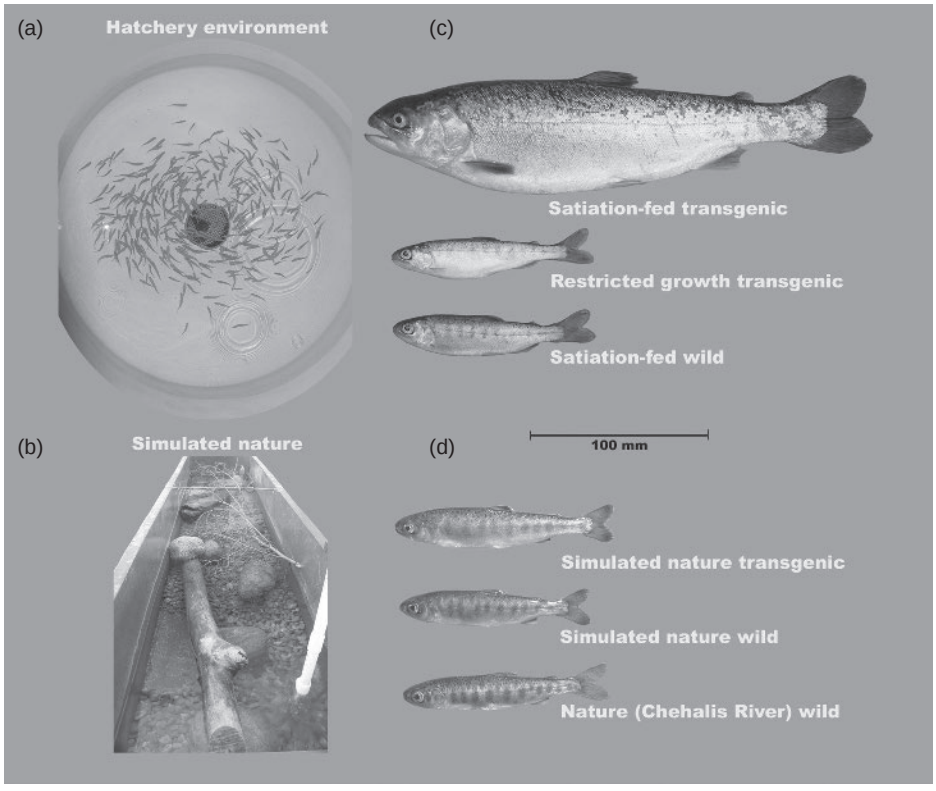

Figure 9.2. Effect of rearing environment on growth of GH transgenic coho salmon. Transgenic fish reared in culture tank environments (a) with satiating levels of artificial feed provided (c, top fish) grow much more rapidly than nontransgenic (c, bottom fish) or ration-restricted transgenic fish (c, middle fish). In seminatural environments (b), that support growth of nontransgenic fish (d, middle fish) as occurs in nature (d, bottom fish), transgenic salmon (d, top fish) do not grow nearly as fast as under culture conditions. (Modified from Sundstrom et al. 2007b.)

wild type, but when both genotypes had been reared in the stream environment from early life, there was much less of a difference in predation effects.

The consequence of a transgenic organism to an ecosystem depends on its specific phenotype produced by the G×E interaction. In most cases, transgenic organisms are characterized as specific strains developed and maintained in the laboratory, often as inbred lines. As such, their phenotypic characteristics may also be specific to those strains. For transgenic fish, characterized lines to date appear to be very stable both phenotypically and in terms of transgene structure (see above) (Nam et al. 2002; Uh et al. 2006; Yaskowiak et al. 2006). However, some evidence suggests that a transgene in fish strains with different genetic backgrounds may respond differently (Devlin et al. 2001). If a transgene enters a population in nature, in many species it would be expected to encounter a diversity of genetic backgrounds resulting in a range of different phenotypes. These different phenotypes would be subject to differential selection and would potentially cause different ecological consequences as well. Thus,

for risk assessments which require prediction of transgene fitness, it is critical to determine whether selection of the transgene is stable or can shift across populations and over time. Furthermore, since differences in phenotype are observed between hemizygotes and homozygotes in some strains (Martinez et al. 1999; Nam et al. 2002; Rosa et al. 2008), the potential for interbreeding with wild conspecifics in nature may determine whether one or both types must be evaluated (i.e., if only homozygotes exist and no wild conspecifics are around to produce hemizygous fish). A promoter may also have different effects depending on species, enhancing growth in some (Devlin et al. 1994; Pitkänen et al. 2001) and having no effect in others that instead are responsive to other promoters (Rahman et al. 1998).

Biocontainment Approaches

Uncertainty associated with environmental risk assessments has highlighted the importance of confinement for aquaculture of transgenic fish. Whereas land-based facilities can be made essentially "escape proof," net-pen facilities in lake or ocean environments (commonly used for salmonids) have historically been associated with escapes of stochastic magnitude and frequency (Naylor et al. 2005). For example, in British Columbia, escapes of Atlantic salmon are sporadic but can be as large as 20,000 individuals per escape (http://www-sci.pac.dfo-mpo.gc.ca/aquaculture/aswp/default_e.htm). Thus, biological methods of containment have also been explored with the objective of reducing the potential for escaped fish to survive in nature and/or to reproduce with themselves or conspecifics to allow persistence of the transgene (Devlin and Donaldson 1992; NRC 2004). Sterilization of finfish can be accomplished in many species through the induction of triploidy by pressure or temperature shocking eggs shortly after fertilization. Both male and female triploids are functionally sterile; however, the former undergo sexual maturation but produce aneuploid sperm and hence the use of all-female triploids is preferred (Benfey 1999). Of critical importance for biocontainment is the frequency of failure of the sterilization technique. Triploidy has been reported to be 100% effective by some commercial sources proposing to use transgenic technology in aquaculture; however, the literature and our experience have revealed that often less than complete triploidy is achieved when adequate numbers of fish are examined. It is known that varying induction parameters can considerably influence the rate of triploidy induction (Cai et al. 1989; Thorgaard 1991), and thus new protocols will likely be needed for each transgenic species/strain used. As an alternative to direct induction of triploidy, the use of tetraploids (which produce diploid gametes) in crosses with diploids (homozygous for a transgene) could potentially yield transgenic triploids with high frequency (Chourrout et al. 1986; Thorgaard et al. 1990). However, tetraploids are difficult to maintain, and can yield diploid and haploid gametes (Chourrout et al. 1986; Nam and Kim 2004), which could be problematic for large-scale application of this method for biocontainment.

Since it is likely that no single method will provide complete containment, additional methods are being explored that can be coupled with current approaches such as triploidy (Devlin and Donaldson 1992). For example, if a diploid hybrid combination is known to be sterile, then such a cross could be used for biocontainment. If an interspecies cross is inviable, then the triploid hybrid variant may possess enhanced survival and be sterile (Grey et al. 1993). In this case, failure of triploidy induction

would be backed up by hybrid inviability as a second containment control. Other approaches (hormonal or chemical sterilization, autoimmune approaches, irradiation) have not proven fully reliable to date (Devlin and Donaldson 1992; NRC 2004).

Transgenic biocontrol methods being explored for fish include both hormonal and cellular approaches. These approaches must be able to be conditionally applied so that production animals can be rendered inviable or infertile if they escape from production facilities into nature, but allow fertile and viable broodstock to be maintained in high-security (e.g., land-based) facilities to allow strain propagation. In some cases, sterilization or inviability would be overcome through application of an exogenous compound that has been made deficient (e.g., hormone and nutrient). For cellular approaches, it may be necessary to conditionally express or suppress the transgene (e.g., using inducible promoters such as Tet-on/off) in broodstock or production fish. Combinations of gene constructs can also be coupled by crossing different strains that, together versus apart, allow for conditional control of transgene expression or effect.

Antisense expression of gonadotropin release hormone (GnRH) constructs in transgenic fish has resulted in partial sterilization of males, presumably through disruption of the endocrine control of gonadal maturation (Uzbekova et al. 2000b; Hu et al. 2007). Rescue of such sterilized fish could in theory be accomplished by administering supplemental GnRH or gonadotropin protein (compounds currently widely used in aquaculture to control maturation timing). The incomplete effects of this antisense method to date may arise from redundancy of GnRH genes in fish genomes that are able to physiologically complement the activities of the targeted gene, or from incomplete suppression of expression by the antisense construct. Many other genes essential for reproductive developments could serve as targets as well, including gonadotropin beta subunits (for LH and FSH), enzymes in the sex steroidogenic pathways, or gonad or gamete structural components (e.g., egg shell or sperm membrane proteins).

In zebrafish, ablation of PGCs by expression of a cytotoxic factor (from a germ cell-specific promoter) results in complete sterility (Slanchev et al. 2005). This approach, albeit effective, may cause significant issues for aquaculture regarding consumer perception and acceptance since the transgene contains a coding region for a cytotoxic protein. Most transgenic approaches for containment carry with them the concern that the sterilization construct itself could induce environmental impacts, should it inadvertently be released to nature. To overcome this in transgenic plants, transgene excision systems have been proposed (Srivastavaa and Owb 2004) and tested with some success. Indeed, application of Cre-mediated gene excision approaches in plants has proven highly effective with failure rates of gene excision less than 0.024% (Mlynárová et al. 2006). Wong and Van Eenennaam (2008) have explored a zebrafish transgenic system where the transgene, flanked by loxP recombination recognition sites, is excised from germ line cells by expression of the Cre recombinase enzyme. Demonstration of tissue-specific (muscle) gene excision by Cre recombinase has been demonstrated in zebrafish (Pan et al. 2005), and Thummel et al. (2005) have shown that conditional expression of Cre from a heat shock promoter can effectively induce gene excision. Use of this approach in commercial fish species could allow for somatic expression of a transgene to induce desired phenotypic changes without concern for the transgene to be transmitted into natural populations in the event of an escape.

Animal Welfare

Welfare issues associated with transgenic fish will be as important as for any other species in aquaculture (Conte 2004). However, little attention has been specifically devoted to better understanding the effects of transgenes and rapid growth on fish welfare. Some welfare issues have been noted, including morphological abnormalities in the head region that may impact feeding, respiration, vision, and swimming (see above). More occurrences of minor fin and eye damage were also noted in transgenic coho salmon relative to wild type (Leggatt et al. 2003). Other welfare aspects include rearing water quality and fish density if transgenic fish can endure lower oxygen levels than wild type (Dunham et al. 2002a) or are more sensitive to low oxygen levels (Cook et al. 2000c; Sundt-Hansen et al. 2007). Similarly, a reduction in ammonia excretion could reduce both nitrogen pollution and increase density at which fish can be reared (Krasnov et al. 1999; Kobayashi et al. 2007), which in turn may alter the probability of pathogen transfer and stress. Transgenic fish have been found to have a better nonspecific immune system (Wang et al. 2006) in one case, but an impaired nonspecific immune system in another case (Jhingan et al. 2003), with both displaying similar learned immune responses as control fish. Transgenic fish may also have upregulated systems to combat oxidants (Leggatt et al. 2007), and the stress response can be affected (Jhingan et al. 2003; Deitch et al. 2006). An important study would be to evaluate whether anticipating food delivery is more stressful for transgenic fish compared to wild-type fish since the former are strongly motivated by appetite whereas the latter are highly influenced by fear.

Societal and Industry Views Affecting Implementation

A great deal of scientific and popular literature has been written regarding the potential hazards of transgenic fish (e.g., Reichhardt 2000; Stokstad 2002), despite no evidence of this being a reality at the present time. As mentioned above, concern centers primarily on environmental risks, but stems from a wider unease with practices that manipulate life for other than critical (i.e., life-sustaining) purposes. Indeed, complex ethical issues are involved stemming from religious beliefs and secular concerns. Clearly, for a technology to be successfully adopted by the aquaculture industry, the public must accept it and purchase and consume its product. End-user acceptance of biotechnology varies considerably by application area and by world geography (Figure 9.3) (Devlin et al. 2004a). For example, biotechnology in general shows a public disapproval rate of approximately 25–45% in developed countries, but only 15% in developing countries. Medical biotechnology is widely accepted by most, due to perceived personal benefits overriding other ethical concerns, whereas less acceptance is observed for crop biotechnology and even less so for animal biotechnology (principally for ethical and food safety reasons). Of relevance to aquaculture, fish biotechnology shows the lowest acceptance rate of all areas being considered. The low tolerability for fish biotechnology may stem from several factors including concern over potential environmental impacts, and that fish are often viewed as pristine environmental entities that should not be manipulated (a view at odds with the massive harvesting of global fish populations that occurs). It is also noteworthy that much greater public acceptance of all biotechnologies, including fish, is observed in developing countries

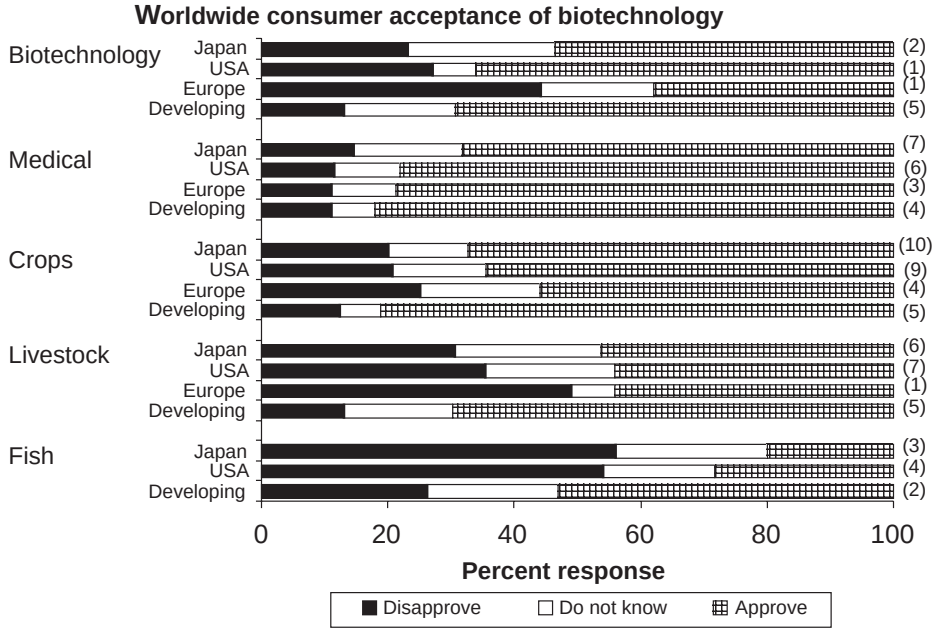

Figure 9.3. Public views of biotechnology associated with different sectors (medical, crop, animal, and fish) in different countries. Data summarized from Anonymous (1993, 1997, 2000, 2001, 2003), Gaskell et al. (1999), Hansen and Nascimento (2003), Hoban (1994, 1996a, 1996b, 1999, 2001), Hoban and Kendall (1993), Li et al. (2003), Macer (1992, 1994, 2000), Macer et al. (1998). (Modified from Devlin et al. 2004a.)

where the need for enhanced food production might benefit most strongly by application of this technology. The pressures to enhance food resources can be expected to influence the level of risks acceptable with regard to food safety and potential environmental impacts. Nongovernmental organizations have played a major role in shaping public opinion surrounding genetically modified organisms, including fish, and industry views mirror public perception closely in developed nations. For example, the North Atlantic Salmon Conservation Organization has resolved that "In view of the current lack of scientific knowledge on the impact of transgenic salmonids on wild salmon stocks, the use of transgenic salmonids should be considered a high-risk activity. There should be a strong presumption against any such use" (NASCO 2006). Whether adoption of transgenic technology to enhance production in one jurisdiction would drive use elsewhere will strongly depend on consumer acceptance in specific markets. Labeling laws in some countries do not require genetically modified foods to be identified to the public, which could prompt counterlabeling by detractors of the technology and influence consumer acceptance. We are aware of only one published indication that transgenic fish could be associated with fish farming at this time (Chen et al. 2000).

The application of transgenic fish in the coming years depends on the interplay of several major factors, including proven food safety qualities, enhanced product qualities for the consumer, consumer acceptance of GM animals as food, producer confidence, demonstration of improved production qualities under aquaculture

conditions, and development of reliable methods for predicting and mitigating environmental impacts.

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Chapter 10
Molecular Regulation of Intermediary Metabolism Focusing on Utilization of Dietary Carbohydrates

Stéphane Panserat

Fish Nutrition

Diet Evolution in Aquaculture: Increased Use of Plant Products in Aquaculture

The aquaculture sector continues to grow more rapidly than any other animal food-producing sector worldwide; that is, at an average rate of 8.8% per year since 1970, compared to only 1.2% for capture fishing and 2.8% for terrestrial farmed meat production systems over the same period (FAO 2007). The expanding aquaculture industry requires a concomitant increase in the production of aquafeeds. Fish meal and fish oil are currently the protein and energy sources of choice in many aquafeeds, mainly in those used in the intensive finfish and crustacean aquaculture sectors (Tacon et al. 2006). The amount of fish meal used in aquafeeds increased from 10% of global production in 1988 to 46% in 2002 (Tacon et al. 2006) and is expected to increase to about 70% by the year 2015 (New and Wijkstroem 2002). However, despite the increase in demand for fish meal, global fish meal production has remained relatively static over the years, fluctuating between 4.57 million tonnes in 1977 and 5.52 million tones in 2003 (Tacon et al. 2006), and there is no evidence to suggest that it will increase in the future. The increase in demand for fish meal compared to supply has therefore led to escalating prices. The dependence of the aquaculture industry on the marine capture fishing sector, and the price and quality fluctuations, have led to the identification, development, and use of alternative ingredients to fish meal. These ingredients are generally classified as those of terrestrial animal origin or plant origin. However, although the production of terrestrial animal by-products (15–30 million tonnes per year, dry basis) exceeds that of fish meal (Tacon et al. 2006), their potential use in aquafeeds in Europe has been severely affected by bovine spongiform encephalitis. Therefore, the main emphasis has been placed on the use of sustainable plant products.

There is intense research activity to find ways to replace marine feedstuffs (fish meal and fish oil) with plant feedstuffs (Gatlin et al. 2007). In particular, in the past 20 years diets have included large amounts of fish oil because the addition of dietary lipids results in less nitrogen waste, thus leading to reduced fish meal input in fish diets and hence reducing pollution. However, total replacement of fish oil by vegetable oils has to date been the main aim of many research projects (Sargent and Tacon 1999). Similarly, partial or total replacement of fish meal by vegetable

plant proteins has recently been widely studied with different fish species. Nitrogen and phosphorus pollution from fish farming can also be reduced by plant-based eco-friendly diets (New and Wijkstroem 2002; Mente et al. 2006). Compared to other alternative protein sources, such as terrestrial animal meal, plant products (e.g., grains, oilseeds, and legumes), have the advantage of presenting relatively constant nutritional composition, greater availability, and more competitive prices (Gatlin et al. 2007).

Understanding of the Nutritional Regulation of Metabolism Is Required to Develop New Diets for Aquaculture

Substantial efforts have been made to evaluate the potential use of plant products in aquaculture diets (Gatlin et al. 2007). However, several disadvantages of using plant feedstuffs in fish diets have been reported such as their relatively low protein content, amino acid imbalance, low palatability, presence of endogenous antinutritional factors (e.g., protease inhibitors, lectins, non-starch polysaccharides, and antigenic compounds), and large amounts of carbohydrates (Gatlin et al. 2007). Replacement of marine resources by plant products in fish diets has certain negative consequences on the quality, health, and growth of farmed fish. For example, replacement of fish oil by vegetable oils drastically reduces the *n*-3 polyunsaturated fatty acid (eicosapentaenoic acid and docosahexaenoic acid) contents that are highly recommended in fish food for human health (Bell et al. 2001, 2002, 2003, 2004). Moreover, total replacement of fish meal by plant proteins seems to be linked to lower growth performance in rainbow trout, linked to changes in a number of hepatic metabolic pathways such as those involved in energy generation (Vilhelmsson et al. 2004). In gilthead sea bream, lower growth has been linked to decreased feed intake (Gomez-Requeni et al. 2004). Finally, most carnivorous fish species do not use dietary carbohydrates efficiently (Hemre et al. 2002). Further investigations into the regulation of metabolism in fish nutrition are therefore essential to provide greater understanding of the use of nutrients.

Effects of Nutrients on Gene Expression of Proteins Involved in Metabolism in Fish

There is growing recognition that dietary micro- and macronutrients are potent influences on the metabolic programming of cells and have an important role in the control of homeostasis, growth, and development. The major reactions of the biochemical pathways leading to metabolism of essential nutrients are relatively well understood, and this has mostly been achieved by studying the substrates and products of reactions and the enzymes catalyzing them. Regulation of reactions has moreover focused on the activity and specificity of the enzymes in terms of allosteric control and posttranslational changes. One way to study changes in metabolic flux through a particular metabolic pathway in response to changes in diet would be to measure substrate and product concentrations of a particular reaction. However, this is not feasible on a large scale for multicellular organisms. Another approach is through analysis of genes that encode these enzymes using recent developments in genomic

technology. Although modifying enzyme levels may not necessarily result in changing metabolic flux through the pathway, significant changes in the expression of the enzyme-encoding genes may reflect metabolic flux change responses. In addition, this approach may identify molecules that regulate specific metabolic pathways, such as transcription factors or components of signal transduction cascades.

Nutritional genomics (nutrigenomics) is the term given to research that investigates interactions between nutrition and the genome. This research is well developed in humans for the evaluation of foods and nutritionally bioactive compounds to promote health and prevent disease (Gillies 2003; Muller and Kersten 2003; Kaput and Rodriguez 2004). It is important to recognize that, in contrast to specific drugs, nutrients can have a number of direct and indirect effects on gene expression. Indeed, organisms have to process a large number of different nutrients that can reach high intracellular concentrations. Each nutrient can also bind to numerous targets with different affinities and specificities. Nutrients may interact with transcription factors or regulate transcription factors to control gene expression. Detailed information on such regulation has been provided by Muller and Kersten (2003).

The classical molecular techniques used to study gene expression are hybridization-based approaches such as Northern blotting, in situ hybridization, and real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) (Figure 10.1). These techniques are highly informative and provide reliable information regarding gene expression, each having its own value, strengths, and weaknesses (Reue 1998; Bustin and Nolan 2004). However, these techniques can study only a few identified genes at any one time. Gene expression profiling may now be performed by using microarray technology, which can monitor the expression of thousands of known and unknown genes simultaneously. The technique is based only on simple hybridization of DNA segments affixed to a single nylon filter or glass slide. The use of microarrays for the study of various aspects of fish physiology has seen a spectacular increase in recent years (Douglas 2006). More details regarding DNA microarray technology can be found in Chapter 4. Genomics programs have been initiated during the past 5 years for "model" farmed fish species (Thorgaard et al. 2002; Liu 2003; Rise et al. 2004) such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), striped bass (*Morone saxatilis*), channel catfish (*Ictalurus punctatus*), and Mozambique tilapia (*Oreochromis mossambicus*). Quantitative nutritional requirements are known for all these fish species (National Research Council 1993). Taking rainbow trout as an example, Thorgaard et al. (2002) provided a comprehensive overview of opportunities for exploiting the tools of genomics in several research areas including fish nutrition. Studies based on "omics" approaches have been relatively scarce in fish nutrition to date except for certain cases of fish oil replacement (Jordal et al. 2005) and fish meal suppression (Vilhelmsson et al. 2004), but this will certainly be the future for research in this area.

Focus on Regulation of Intermediary Metabolism by Dietary Carbohydrates in Carnivorous Fish at the Molecular Level

All dietary ingredients (macro- and micronutrients) can clearly have an impact on intermediary metabolism such as lipid metabolism, protein metabolism, or energy metabolism in different tissues (liver, muscle, and fat). These metabolic targets have

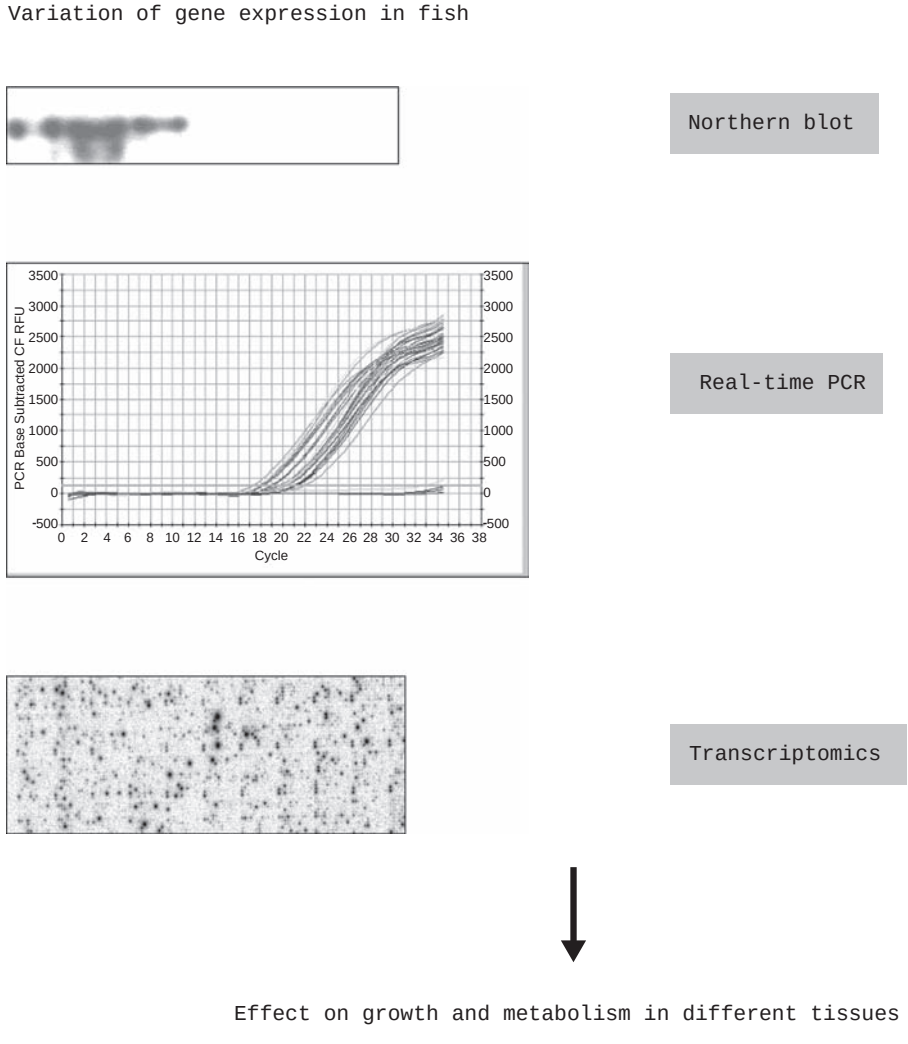


Figure 10.1. Schematic representation of nutrigenomics. Different methods to analyze gene expression linked to the nutritional status are Northern blotting, quantitative RT-PCR, and transcriptomics.

been fully studied in fish, linked to changes in dietary lipid intake and dietary protein intake (Gatlin et al. 2007). The main metabolic pathways studied have been lipogenesis, fatty acid β-oxidation, and lipid biotransformation for changes in dietary lipids, and amino acid catabolism, protein synthesis, and proteolysis for changes in dietary proteins. In this chapter, the effects of another macronutrient and carbohydrates on intermediary metabolism in fish species at a molecular level are analyzed. Although relatively few studies have been undertaken with carbohydrates in fish compared to lipids and proteins, the main focus is currently to understand the reasons for their poor use by carnivorous fish; the molecular approach should help the scientific community to explain the impediment to carbohydrate use in carnivorous fish.

Poor Use of Dietary Carbohydrates by Farmed Carnivorous Fish

Carbohydrates are the main components of grains, legumes, and oilseeds. Cereals and pulses contain 65–75% and 50–60% of their total weight in the form of carbohydrates, respectively, and in roots and tubers carbohydrates comprise about 15–30%. Carbohydrates are generally classified in two types—reserve energy polysaccharides and structural polysaccharides, usually known as non-starch polysaccharides. Starch is the predominant energy storage carbohydrate in cereal grains. Starch constitutes approximately 60 and 70% of the total grain in wheat and maize, respectively, while in legumes starch accounts for about 35–45% of the seed weight, with the exception of the lupin (<0.5%) (Hedley 2001). In soybeans, the most important oilseed, starch accounts for about 1.5% of the seed weight (Hedley 2001). The highest levels of structural polysaccharide compounds are generally found in legume seeds.

Carbohydrates are the most economical energy source for humans and terrestrial animals. However, utilization of carbohydrates by fish is in general inferior to that of domestic animals and varies between fish species (Wilson 1994). It appears that the utilization of dietary carbohydrates is related to the fish digestive and metabolic systems, which are adapted to different aquatic environments, and is affected by carbohydrate level, origin, complexity, and physical state (Wilson 1994; Hamre et al. 2005). Indeed, omnivorous and herbivorous fish such as the common carp (*Cyprinus carpio*), tilapia, channel catfish, and Indian major carp species (catla, *Catla catla*; rohu, *Labeo rohita*; and mrigal, *Cirrhina mrigala*) can be fed high levels of carbohydrates without negative effects on growth and with good protein sparing effects (Wilson 1994). In contrast, growth of carnivorous fish such as salmonids (Atlantic salmon and rainbow trout) and European sea bass (*Dicentrarchus labrax*) is lower when levels of dietary carbohydrates are higher than 20% (Wilson 1994; Hemre et al. 2002): these fish species show persistent postprandial hyperglycemia when fed carbohydrates (Hemre et al. 2002). In the replacement of fish meal with plant feedstuffs naturally rich in carbohydrates in diets for carnivorous fish, it is important to analyze nutritional regulation of glucose metabolism in order to understand why these species have difficulties utilizing high levels of digestible carbohydrates. Various hypotheses (Wilson 1994; Moon 2001) have been proposed to explain interspecies differences in the utilization of dietary carbohydrates. Recent studies in this area of research have proposed molecular approaches to answer some of these questions. The following is an overview of these experiments.

Regulation of Gene Expression of Metabolic Factors by Dietary Carbohydrates in Fish Tissues

After intake of carbohydrates, followed by digestion and absorption of glucose by the intestine, glucose is stored in major tissues (i.e., liver, fat, and muscle) following the action of hormones (mainly insulin and glucagon) on specific metabolic pathways. The regulation of glucose metabolism is often linked to a specific action on gene expression (long-term effect), but many other acute mechanisms such as protein phosphorylation–dephosphorylation, allosteric regulation of enzymes, and intracellular localization of nutrient transporters are also important factors. However, they are not described here because they are outside the remit of the present review.

Molecular Regulation of Digestive Enzymes by Dietary Carbohydrates

As the intestine is the first organ that comes in contact with nutrients, it has a key role in the digestion and absorption of nutrients. As expected, not all fish species have the same capacity to digest complex carbohydrates (Krogdahl et al. 2005). Although all the digestive enzymes are present (mainly of pancreatic origin), at this level their expression can be very different between herbivorous fish and carnivorous fish. Pancreatic amylase, which catalyzes the digestion of starch in the intestine, has been the most frequently studied enzyme at a molecular level in terms of its regulation by feeding. Amylase is more highly expressed in adult omnivorous fish than carnivorous fish (Krogdahl et al. 2005). Amylase has been analyzed just before and after the first feeding in European sea bass at different larval stages. Interestingly, high levels of amylase gene expression have been detected before mouth opening (Zambonino and Cahu 2001). Although gene expression is maintained at higher levels according to dietary carbohydrate content, the level of amylase gene expression decreases significantly according to endogenous feeding stages (Zambonino and Cahu 2001). It is surprising that carnivorous fish larvae show high levels of gene expression of proteins coding for a starch digestive enzyme, whereas the vitellus is almost devoid of glycogen reserve. This has also been reported in freshwater rainbow trout (Geurden et al. 2007).

Molecular Regulation of Metabolic Enzymes in Glucose Sensor Tissues (Pancreas, Hypothalamus) by Dietary Carbohydrates

Food intake and partitioning of nutrients are regulated by specific tissues such as the pancreas and specific regions of the brain (hypothalamus). Control of glucose homeostasis is in fact dependent on pancreatic beta cells, which are responsible for insulin secretion after glucose intake (MacDonald et al. 2005). The brain also regulates energy homeostasis by balancing energy intake, expenditure, and storage (Levin 2001). The glucose phosphorylating enzyme glucokinase has structural, kinetic, and molecular genetic features that are ideal for its primary role as glucose sensor in a network of neuro/endocrine sentinel cells that maintain glucose homeostasis in many vertebrates (Matschinsky et al. 2006). Studies of the dietary responses of these tissues at a molecular level have recently been undertaken in rainbow trout. Soengas et al. (2006) and Polakof et al. (2007) have shown that glucokinase gene expression is induced just after a meal in beta cells and in the hypothalamus in rainbow trout fed with standard diets. These findings demonstrate that carnivorous fish possess signaling pathways involved in glucose sensoring in specific tissues, which are very important for carbohydrate partitioning in liver, muscle, and fat tissues. More studies are required to check the regulation of these pathways in fish species by different levels of dietary carbohydrates.

Molecular Regulation of Glucose Transport by Dietary Carbohydrates

Glucose transport inside cells is the first step in glucose utilization in any organism (Wood and Trayhurn 2003). Studies at the molecular level have distinguished several

distinct types of glucose transporters and detected their presence or absence in fish. The intestinal Na^+-dependent glucose cotransporter was found to be expressed at all developmental stages of rainbow trout (Geurden et al. 2007). Glucose transporter 1 (Glut1, an ubiquitous glucose transporter), glucose transporter 2 (Glut2, a transporter of high glucose concentrations both into the liver/pancreas and out of the intestine to blood), and glucose transporter 4 (Glut4, an insulin-sensitive transporter involved in glucose transport in muscle and fat tissues) have been found in rainbow trout through cloning of the corresponding genes and analysis of their expression (Teerijoki et al. 2000; Capilla et al. 2002). Capilla et al. (2004) reported the cloning of a salmon Glut receptor from adipose tissue structurally and functionally homologous to mammalian Glut4, but with a lower affinity for glucose. The results from Capilla et al. (2002, 2004) and Diaz et al. (2007) indicate that Glut4 mRNA and protein levels in the red muscle of brown trout correlate with levels of insulin in the blood but not in the white muscle. More studies are required to analyze the nutritional control of Glut4 expression, in particular its capacity of translocation to the membrane.

Molecular Regulation of Metabolic Enzymes in the Insulin-Sensitive Liver by Dietary Carbohydrates

The liver plays a key role in coordinating body metabolism in response to nutritional status (Pilkis and Granner 1992; Klover and Mooney 2004). Most of the regulatory effects occur initially in the liver, which then influence the activities of other organs regarding nutrient utilization and metabolism. The metabolic pathways leading to both synthesis and degradation are active in the liver. One of the hypotheses to explain low dietary carbohydrate utilization is atypical regulation of hepatic glucose metabolism in fish fed high levels of carbohydrates. The first metabolic pathway is involved in the storage of end products of glucose utilization (glycolysis, lipogenesis, glycogenogenesis). When mammals are fed carbohydrates, there is induction of the enzymes in these metabolic pathways mainly linked to increases in mRNA levels (Pilkis and Granner 1992). Researchers have found that the first enzyme in glucose phosphorylation, that is, glucokinase, is highly induced in rainbow trout and gilthead sea bream (*Sparus aurata*) after being fed carbohydrates (Caseras et al. 2000; Panserat et al. 2000a) and this is related to higher glucokinase gene expression, as in mammals (Figure 10.2). The cloning of the glucokinase cDNA and its nutritional regulation were the first demonstrations of the possible adaptation of carnivorous fish to carbohydrates by mechanisms similar to those in mammals. The initial hypothesis of the absence of an inducible glucokinase in fish liver (Wilson 1994) was clearly refuted by these studies (at least in these species). The overall findings suggest that this step is not the limiting factor to explain low dietary carbohydrate utilization in fish. The second hepatic metabolic pathway proposed is that corresponding to the production of endogenous glucose. Two metabolic pathways are involved, that is, glycogenolysis and gluconeogenesis. In contrast to mammals (Pilkis and Granner 1992; van de Werve et al. 2000) and gilthead sea bream (Caseras et al. 2002; Panserat et al. 2002b), no decrease was found in glucose-6-phosphatase, fructose-1,6-biphosphatase (FBPase), or phosphoenolpyruvate carboxykinase activity and gene expression regardless of whether the trout were fed with elevated levels of dietary carbohydrates (Panserat

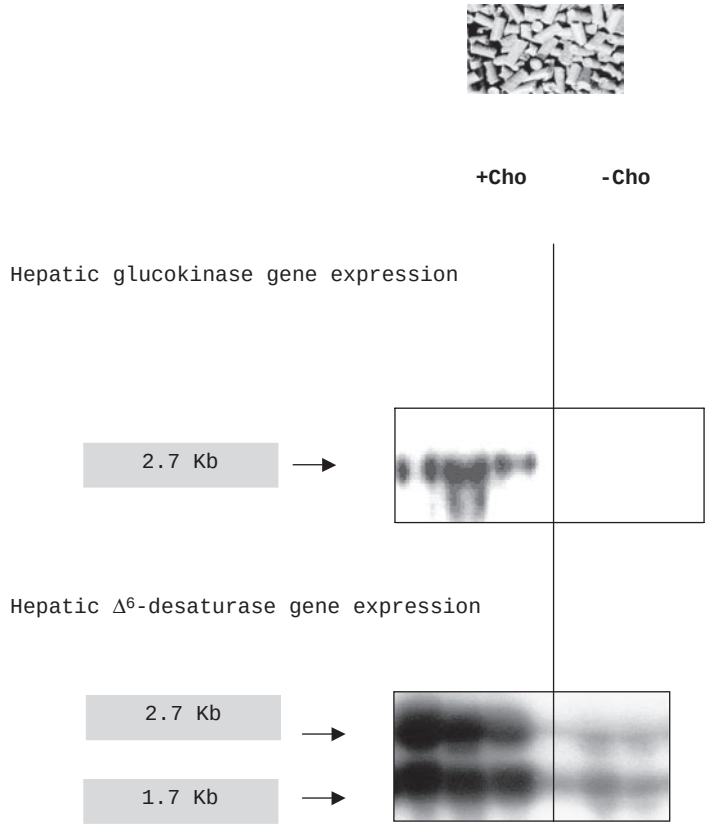


Figure 10.2. Effect of dietary carbohydrates on intermediary metabolism: hepatic glucokinase gene expression and hepatic Δ^6-desaturase gene expression in rainbow trout. (Adapted from Panserat et al. 2000a and Seiliez et al. 2001.)

et al. 2000b, 2001a, 2001b) (Figure 10.2). This suggests that, as in diabetic humans, there is a persistently high level of endogenous glucose production by the liver in the trout, leading to competition between exogenous (dietary) glucose and endogenous glucose as a source of energy. The reasons for the absence of regulation of hepatic gluconeogenic enzymes by dietary carbohydrates are not clear, but may be due to the high levels of dietary gluconeogenic amino acids (main substrates for glucose production) and fatty acids in the diets (Panserat et al. 2002a; Kirchner et al. 2003). Postprandial gluconeogenesis in extrahepatic tissues such as the intestine and kidney may also be very important, as shown by the molecular expression of the gluconeogenic FBPase enzyme in these tissues (Kirchner et al. 2005). Hepatic glycogen metabolism is highly important for storage of excess glucose (glycogenesis) and endogenous production of glucose (glycogenolysis), depending on nutritional status and carbohydrate intake. Clearly, intake of carbohydrate is associated with higher levels of glycogen in the liver (Panserat et al. 2000a). However, key enzymes such as glycogen synthase and glycogen phosphorylase have been studied only at an enzymatic level but never at a molecular level in fish.

We have so far presented the effects of dietary carbohydrates on hepatic glucose metabolism. However, as interactions can occur between all the metabolic pathways, some effects of dietary carbohydrates have been found on other metabolic targets. Indeed, intake of 20% dietary carbohydrates has been reported to induce hepatic gene expression of key enzymes involved in fatty acid biosynthesis (fatty acid synthetase) and fatty acid biotransformation (Δ^6-desaturase) in rainbow trout (Seiliez et al. 2001) (Figure 10.2).

Molecular Regulation of Metabolic Factors in Insulin-Sensitive Muscle and Fat Tissue by Dietary Carbohydrates

Plasma turnover and oxidation are slower in most teleosts than in mammals (van den Thillart 1986). The white muscle in fish represents up to 80% of the weight of the animal and presents a mainly anaerobic glycolytic metabolic pathway, whereas the red muscle and heart are aerobic tissues. One major difference between trout muscle and mammalian muscle is in their ability to utilize blood-borne glucose as a glycolytic and glycogenic substrate. White muscle seems to use low levels of exogenous (extracellular) glucose, preferring the use of lactate or palmitate as an energy source (Frolow and Milligan 2004; Kam and Milligan 2006). Interestingly, glucose transport within the muscle seems to be inefficient although there are insulin receptors and glucose transporters in muscle membranes (Parrizas et al. 1994; Legate et al. 2001). The low number of muscle insulin receptors compared to insulin-growth factor 1 (IGF1) receptors (Parrizas et al. 1994), the low level of glucose transporters (Legate et al. 2001), and the low glucose phosphorylation capacity reflected by the low hexokinase activity (Kirchner et al. 2005) in carnivorous fish may be the reasons for the relative insensitivity of white muscle to exogenous glucose in rainbow trout. Even after 10 days of adaptation with high levels of carbohydrates, there is still a low-glucose metabolism response in white muscle compared to 3 days of adaptation (Table 10.1). Some studies using a molecular approach have reported the low-level presence of a glut4-like glucose transporter in the muscle tissue of salmonids weakly induced by insulin injection (see the previous paragraph). Studies of glycogen metabolism based on key enzymes, for example, glycogen phosphorylase and glycogen synthase, have not been

Table 10.1. Adaptation to 30% digestible dietary carbohydrate (dextrin) in juvenile rainbow trout either after 3 days of feeding or after 10 days of feeding.[1]

	Fish fed for 3 days	Fish fed for 10 days
Glycemia (g/L)	0.9 ± 0.3	3.3 ± 1.1
Muscle glycogen (mg/100 mg)	0.4 ± 0.1	1.0 ± 0.4
Muscle hexokinase activity (mU/mg protein)	1.0 ± 0.2	1.0 ± 0.3
Muscle pyruvate kinase activity (U/mg protein)	4.6 ± 1.3	5.5 ± 0.7
Hepatic glucokinase gene expression (real-time PCR)	No detectable gene expression	High level of gene expression

[1]Parameters (means $\pm$ SD) measured 6 hours after feeding.

evaluated using a molecular approach. Further studies of the nutritional regulation of metabolism at a molecular level are clearly needed to explain the response of white muscle to dietary glucose.

Fat is a major tissue for lipid storage in higher eukaryotes. This lipid pool is in a constant state of flux, resulting from a largely futile cycle of lipolysis and reesterification. However, in contrast to mammals, the liver is the main site for lipogenesis in fish (Hemre et al. 2002). Adipose tissue is also an endocrine tissue (Badman and Flier 2007). It has an impact on glucose homeostasis, especially the perivisceral fat tissue, which is present in large quantities in salmonids. Very few molecular studies have been carried out on this tissue in relation to dietary carbohydrate intake in farmed fish to our knowledge. It seems that adipose tissue does not have a major role in glucose storage in fish.

Determination of the Mechanisms Involved in Molecular Regulation by Dietary Carbohydrates in Fish

Direct (Metabolic) and/or Indirect (Hormonal) Regulation by Dietary Carbohydrates

Regulation of metabolism by dietary carbohydrates is known to be due to direct (through metabolites) and/or indirect (through hormones such as insulin and glucagon) factors (Pilkis and Granner 1992). There is little information regarding the molecular mechanisms of metabolism regulation by dietary carbohydrates in fish. However, preliminary hormonal (insulin) and metabolic (glucose) findings regarding regulation of hepatic glucokinase, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase, and glucose-6-phosphatase gene expression have recently been published for gilthead sea bream (Salgado et al. 2004; Meton et al. 2006; Egea et al. 2007a, 2007b). In vivo and in vitro analysis (in primary cell cultures) is very useful to obtain a clear picture of the role of each of the factors. For example, the glucose-6-phosphatase gene responds in primary hepatocytes in gilthead sea bream as in mammals, both after insulin injection in vivo and after insulin supplementation (Salgado et al. 2004). Other approaches based on the use of drugs and their actions on metabolic pathways are also interesting. Metformin (an antidiabetic drug) has a hypoglycemic effect on rainbow trout fed with carbohydrates through inhibition of hepatic gluconeogenic gene expression, suggesting the significance of gluconeogenesis in poor control of glycemia in fish fed with carbohydrates.

Transcriptional Factors Influenced by Dietary Carbohydrate Intake

Complex networks of transcriptional factors are involved in dietary glucose-regulated genes. Among the major factors known to be influenced by dietary carbohydrates and insulin are the carbohydrate responsive element-binding protein (ChREBP), sterol-regulatory-element-binding protein-1c (SREBP-1c), liver X receptor (LXR), peroxisome-proliferator-activated receptor-gamma coactivator-1 alpha (PGC1α), hepatocyte nuclear factor (HNF4), and forkhead protein (FOXO1) (Barthel and Schmoll 2003; Postic et al. 2007). Preliminary research into the actions of the

transcription factors (SP1, SP3, Srebp1a, HNF4, PGC1α) on glucose enzyme promoters has recently been published for gilthead sea bream (Salgado et al. 2004; Meton et al. 2006; Egea et al. 2007a, 2007b). PGC1α, a key factor involved in positive regulation of gluconeogenic genes in mammals, seems to be paradoxically highly expressed in fed rainbow trout compared to fasted fish, and this could explain the absence of inhibition of gluconeogenic gene expression. These results describing the role of molecular factors in gene expression raise new research issues to explain and resolve the low use of carbohydrates by carnivorous fish. The existence of nucleotide databases containing sequencing (and annotation) of the transcriptome (all the mRNA), combined ultimately with the sequence of the genome of some fish species, will considerably assist research into the identification of all these factors in fish metabolism.

What Can Be Done to Improve the Use of Dietary Carbohydrates by Farmed Carnivorous Fish?

Through Genetic Selection

One powerful but time-consuming approach to improve fish nutrition is to study/use the natural genetic polymorphism of fish with nutritional parameters such as feed intake, feed efficiency, and lipid storage (Mambrini et al. 2004; Quillet et al. 2005; Kause et al. 2006). Improving the use of specific and alternative nutrients by fish could be linked to higher or lower levels of specific gene expression. Using resource families (Mambrini et al. 2004; Quillet et al. 2005) and DNA markers from genomics sources (Liu and Cordes 2004), it is expected that greater success will be achieved for marker-assisted selection in the near future. However, no known experiments to select carnivorous fish for the ability to use greater levels of carbohydrates have been initiated.

Through Transgenesis

The first transgenic fish was reported in 1985 (Zhu et al. 1985). Much research is currently being undertaken with a number of teleosts (salmonids, cyprinids, catfish, tilapia, etc.) (Sin 1997). Germ-line transgenic fish have been mainly produced by microinjection of gene constructs into the fertilized egg shortly after fertilization (Sin 1997). This technology is rapid and easy, due to the transparency and large size of most fish eggs (however, this is not the case for all fish species). The major inconvenience of this approach is the low efficiency of transgenesis. Production of transgenic fish offers a valuable means of studying gene function because it allows the detection of phenotypes that have been changed by a gain in function. Transgenesis has rarely been employed to date to study fish carbohydrate nutrition directly, except in one case of improved dietary glucose utilization by overexpression of Glut1 and hexokinase II in rainbow trout (Krasnov et al. 1999; Pitkanen et al. 1999). No improvement in postprandial glycemia or higher levels of exogenous glucose transport in muscle was observed. However, the results were far from convincing due to technological issues, including high levels of mosaicism and absence of expression of the transgenic product. Moreover, in order to produce transgenic fish with the relevant transgene to improve

glucose use, it is very important first to understand the precise reasons for the poor use of dietary glucose in these fish species.

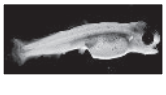
Through Nutritional Programming

Several studies in mammals and humans showed that dietary influences exerted at critical developmental stages early in life may have long-term consequences on physiological functions in later life (Lucas 1998). Possible biological mechanisms for the nutritional programming event until adulthood include adaptive changes in gene expression (epigenetic phenomenon), preferential clonal selection of adapted cells

(a)

Genes	**Fed larvae (3 days) compared to unfed larvae**
SGLT1	2.5 upregulation
Glucokinase	Switch on
Glucose-6-phosphatase	2.1 downregulation

(b)



Genes	**Type of regulation**	***P*-values**
(i)		
α-Amylase	upregulation—4-fold	<0.05
Maltase	upregulation—2.2-fold	<0.05
(ii)		
α-Amylase	upregulation—1.7-fold	<0.05
Maltase	upregulation—1.5-fold	<0.005

Figure 10.3. (a) Regulation of glucose metabolism during the endogenous–exogenous transition. (b) Nutritional programming of the enzyme gene expression in rainbow trout: (i) comparison between larvae fed hyperglucidic diet and commercial diet at first feeding and (ii) comparison between fish previously fed with hyperglucidic diet and those fed with commercial diet for juvenile fish fed with 25% of carbohydrates. (Adapted from Geurden et al. 2007.)

in programmed tissues, and programmed differential proliferation of tissue cell types (Lucas 1998). Changes in adult glucose metabolism due to early nutritional events have been reported in several studies. Temporary exposure to increased levels of insulin during gestation was shown to cause glucose intolerance in rat progeny, whereas prenatal dietary protein restrictions induced lifetime changes in hepatic glucose metabolism (glucokinase and phosphoenol pyruvate carboxykinase activity) (Desai et al. 1995). Based on the concept of nutritional programming in higher vertebrates, researchers recently investigated whether an acute hyperglucidic stimulus during early life could induce a long-lasting effect on carbohydrate utilization in carnivorous rainbow trout. The rainbow trout were fed a hyperglucidic diet (60% dextrin) at first feeding (3 days). Prior to and after the hyperglucidic stimulus, they received a commercial diet until the juvenile stage. In the final challenge test with juveniles fed a 25% dextrin diet, both digestive enzymes were upregulated at the molecular level in fish exposed to the hyperglucidic stimulus at first feeding (Figure 10.3), confirming the possibility of modifying certain physiological functions in rainbow trout in the long term (Geurden et al. 2007). The absence of harmful effects of these stimuli on fish growth and their survival highlight a promising new area of study in the investigation of nutritional programming of glucose utilization in fish, and this is of practical relevance for aquaculture.

Conclusion—Perspectives

In conclusion, this provides an overview of the powerful possibilities of using molecular biology in the context of fish nutritional metabolism. The development of such approaches in combination with others, that is, classical (measurement of nutritional parameters), biochemical measurements, cell signaling, proteomics, and metabolomics, will help the development of eco-friendly and sustainable fish nutrition in aquaculture.

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Chapter 11
Muscle Regulation

Peggy R. Biga

Muscle physiology research has mainly focused on mammalian animal models, but more recently the focus on understanding muscle development has led to increased investigations into fish muscle regulation. One reason for this focus is based on the importance of fish as a high-quality source of protein for human consumption and possible increased health associated with higher fish consumption. Other reasons include the interesting physiological differences that exist between aquatic and terrestrial or between fish and mammal vertebrates. The same basic genes are present and appear functional; however, slightly different regulatory mechanisms exist in the regulation of fish muscle development and growth. The most notable difference occurs in postembryonic myoblast proliferation and differentiation.

The zebrafish (*Danio rerio*) has become an important biomedical model organism, particularly for developmental research. The zebrafish possess many desirable traits for biomedical research, including a short generation time of 3 months, external development of clear embryos, and a small adult size that allows for large-scale experiments in small laboratory spaces (Kimmel 1989; Higashijima et al. 1997). With the aide of the sequenced zebrafish genome, large-scale mutagenesis screenings have led to the identification of many genes important in embryonic development (Driever et al. 1996; Haffter et al. 1996; Amsterdam et al. 1999). Muscle development research has utilized the zebrafish extensively to understand cell differentiation and migration. Therefore, much of the muscle regulation and development research covered here focuses on work conducted in zebrafish.

This chapter focuses on the current knowledge of myoblast proliferation, detailing differences between mammalian and piscine muscle growth and proliferation regulation. Current molecular and physiological techniques used in fish muscle growth research are detailed and compared to the current mammalian literature. Also, the importance of this scientific information to the aquaculture industry is discussed. This chapter also contains points of interest for future directions that research in fish muscle growth and development might focus.

Muscle Physiology

All vertebrates possess skeletal muscles that function in movement, stability, temperature control, and posture. Each muscle forms a distinct organ with a complex organization highly specialized for physiological responses (Huxley 1965; Kushmerick et al. 1992; Pette and Staron 1997). All skeletal muscles contain multinucleated fibers that arise from embryonic muscle cells called myoblasts. Myoblasts fuse during embryonic development to form a myotube that will "mature" into a muscle fiber. The

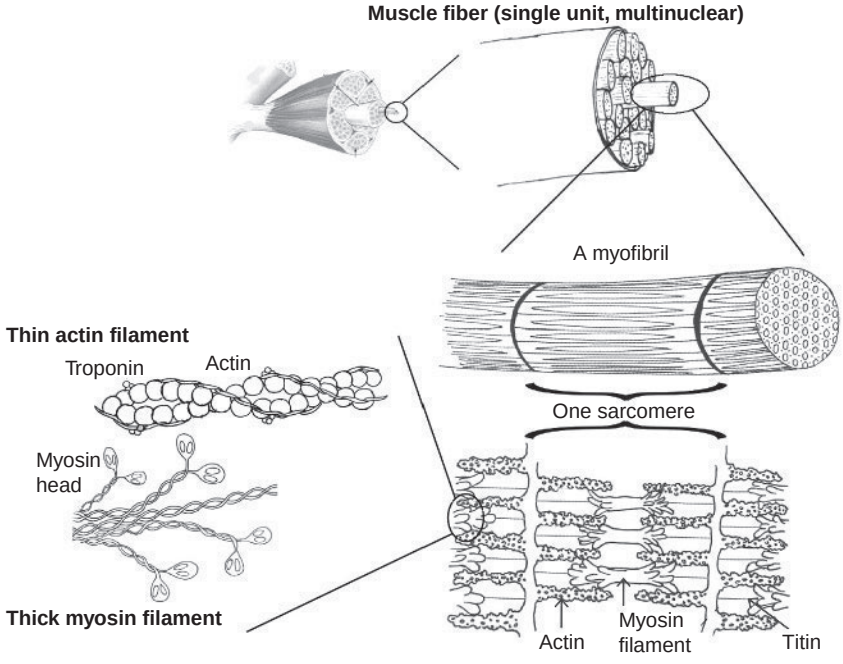

Figure 11.1. Components and makeup of muscle fibers. The muscle fiber cell, single unit, and its connections with other muscle cells. The myofibril component of the muscle is made up of sarcomeres, with basic components including the actin thin filament and the thick myosin filaments.

fibers are composed of myofibrils, sarcoplasmic reticulum, T-tubules, and mitochondria (Figure 11.1). The myofibrils contain the actin and myosin filaments that form the contractile unit, or sarcomere, of the muscle. Myosin filaments are phenotypically diverse and vary depending on the muscle fiber type and developmental stage.

Skeletal muscle fibers are classified based on two functional characteristics: contractile speed and metabolic activity (aerobic: oxidative or anaerobic: glycolytic). These two functions are interrelated, as the speed of contraction reflects how fast and by which physiological mechanism the cell metabolizes ATP. Therefore, fast fibers (type IIb or white fibers) utilize anaerobic glycolysis and slow fibers (type I or red fibers) utilize aerobic oxidative phosphorylation. Fast fibers are characterized by containing small amounts of mitochondria and myoglobin, but are rich in glycogen, making them suitable for intense short-burst activity. In contrast, slow oxidative fibers are high in mitochondria and myoglobin, making them suitable for longer sustained activity, or endurance activity. Pink fibers are considered intermediate (type IIa), with fast-twitch capacity combined with aerobic metabolic capacity to resist fatigue.

Different fiber types exhibit varying morphological and metabolic properties, including fiber diameter and area, contraction rate (V_{max}), mitochondria content, oxidative phosphorylation capacity, and anaerobic glycolysis enzymes (Table 11.1). Myosin ATPase (mATPase) activity also varies between fiber types and provides a histological technique to distinguish fiber types (Figure 11.2). Vertebrate skeletal muscles typically contain more than one type of muscle fiber, proportionate to the properties of physical

Table 11.1. Varying morphological and metabolic properties exhibited by different fiber types.

Property	Slow oxidative (type I)	Fast oxidative (type IIa)	Fast glycolytic (type IIb)
Fiber diameter	↓	←→	↑
Fiber cross-sectional area	↓	←→	↑
Rate of contraction (V_{max})	↓	↑	↑
Myosin ATPase activity	↓	↑	↑
Mitochondria number	↑	↑	↓
Oxidative phosphorylation capacity	↑	↑	↓
Anaerobic glycolysis enzymes	↓	←→	↑

Adapted from Sherwood (2001) and Randall et al. (2002).
Key: ↓, low; ↑, high; ←→, intermediate.

use of the particular muscle. However, in most teleost fish slow and fast fiber types are arranged in anatomically discrete layers (Figure 11.3). This muscle fiber arrangement makes fish a great model organism for studying vertebrate myogenesis.

Fiber organization also differs between fish and mammals. Mammalian muscle is based on a single muscle fiber, which is very long relative to its width. Bundles of fibers are surrounded by connective tissue to form primary bundles that are grouped to form whole muscles with specialized functions (Romans et al. 1985). In fish, the

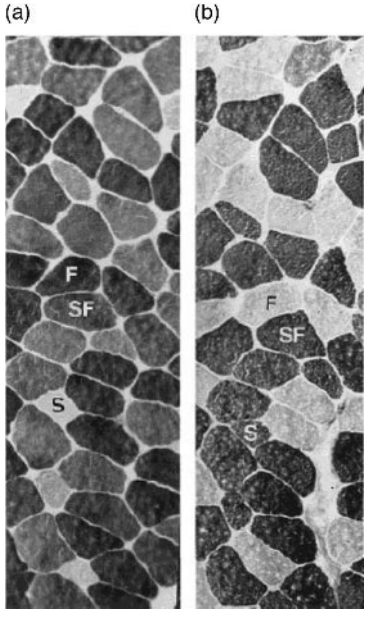

Figure 11.2. Myofibrillar ATPase staining in serial sections from masseter of a 16-week pig. (a) Alkaline stable and (b) acid stable (×400 magnification). F, fast-twitch fiber cells; S, slow-twitch fiber cells; SF, intermediate fibers exhibiting both acid- and alkaline-stable mATPase. *Source*: Anapol and Herring (2000).

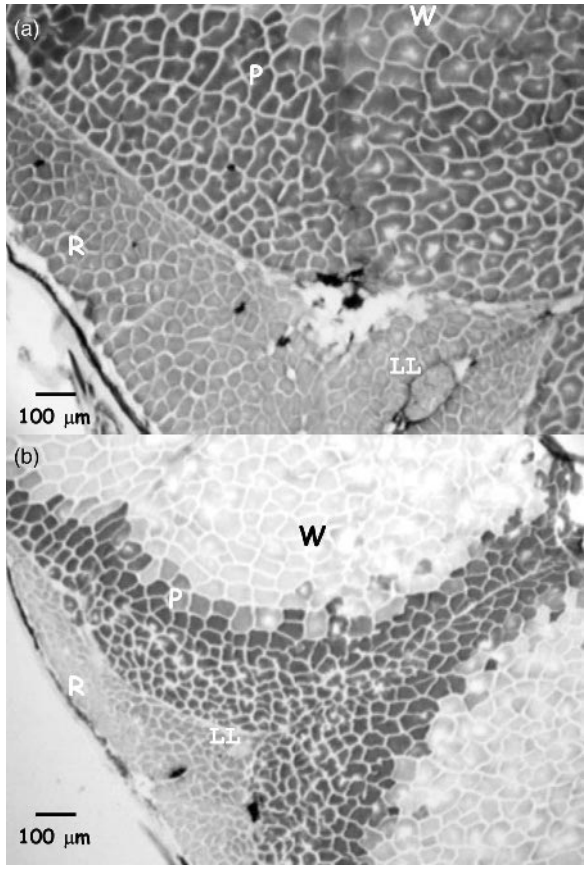

Figure 11.3. (a) Giant danio and (b) zebrafish cross sections. mATPase staining, acid stable. P, pink fiber cells; W, white fiber cells; R, red fiber cells; LL, lateral line. Scale = 100 μm, ×40 magnification (Biga and Goetz 2006).

organization is based on single fibers like in mammals, but the organization of the whole muscle is much more simple. However, the single fibers are surrounded by myocommata to form a myotome that is considered to be the whole muscle of the fish (Love 1970). This is in contrast to the bundles of fibers comprising functional muscle groups in mammals. The size of individual sarcomeres is also different between fish and mammals. In fish the "I" band is approximately half the width of the "A" band (Love 1970), while in mammals the "I" band is approximately two-thirds the width of the "A" band (Romans et al. 1985). The functional significance of the bandwidths is still unclear, but is likely related to contraction force.

The muscle fibers of most fish are arranged in a unique manner that facilitates form and function within their environment. The swimming muscle, which constitutes almost 60% of total body mass, consists of repeating near-identical units called myotomes. The myotomes are arranged in a chevron shape and are separated by thin connective tissue sheets called myosepta (Figure 11.4). To facilitate propulsion and body undulation, the myosepta transmits the force of contraction to the axial skeleton

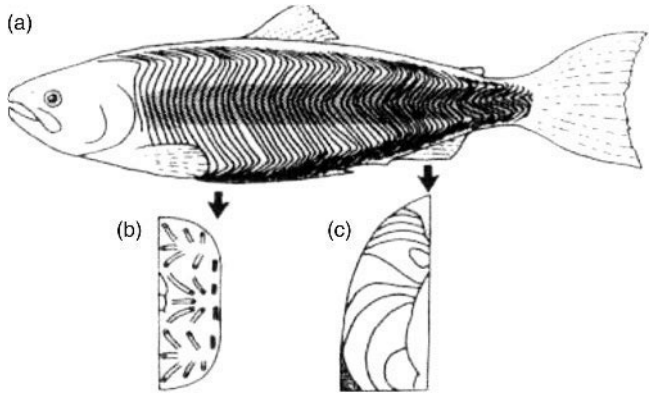

Figure 11.4. (a) Demonstrates the chevron-shaped myotome of fish musculature. (b) Illustrates the distribution of muscle fibers in a transverse section through the caudal myotomes. (c) Demonstrates the orientation of muscle fibers in a transverse steak (based on Alexander 1969). (Taken from Kestin and Warris 2001.)

and caudal fins through tendons (Videler 1993). The horizontal septa and lateral line divide the epaxial and hypaxial segments of the myotome. The bulk of fish musculature, all segments, consists of predominately fast glycolytic fibers that are poorly supplied with capillaries, mitochondria, and low activities of oxidative enzymes (Bostrom and Johansson 1972; Hamoir et al. 1972; Mattisson et al. 1972; Patterson and Goldspink 1972). A superficial layer beneath the lateral line consists of slow oxidative fibers in most fish species (Figure 11.3). These fibers are rich in capillaries, mitochondria, and highly active oxidative enzymes (Bostrom and Johansson 1972; Mattisson et al. 1972; Patterson and Goldspink 1972).

In teleosts, it is common for the red muscle layer to be confined to a superficial layer lateral to the horizontal septum, which is significant for more constant tailbeat frequency. However in some fishes, like adult scombroids (tunas and billfishes), red fibers incorporate and internalize into the deep fast-glycolytic muscle zone (Carey 1973; Block et al. 1993; Altringham and Block 1997). Tunas are unique among teleosts, as they are endothermic and are large pelagic, continuously swimming fish. These unique properties are interesting, as the endothermy is compartmentalized in regions of high metabolic demand, such as the slow musculature which is responsible for continuous swimming. The compartmentalization is characterized by modified vascularization that forms heat exchangers, resulting in the ability to retain heat and providing an increase in muscle performance at temperatures ranging from 17.5 to 28°C (Block 1991; Altringham and Block 1997).

As previously mentioned, most teleosts exhibit a superficial layer of red muscle, with a much larger mass of white fibers beneath the superficial layer and lateral line. Red fibers are smaller in diameter, are relatively homogeneous in size, and exhibit aerobic metabolism and low mATPase activity (Johnston et al. 1972, 1974, 1975a, 1975b; Carpene et al. 1982). In comparison, white fibers are larger in diameter, more heterogeneous in size, and exhibit glycolytic anaerobic metabolism with high mATPase activity (Carpene et al. 1982; Johnston 1982). The proportions of slow and fast muscle fibers are not consistent or uniform along the body length of all fish. In

nonendothermic fishes, the amount of slow fibers increases caudally toward the tail, to aid in bending of the caudal fin (Rome et al. 1992a, 1992b, 1993).

Also, at hatching, the distribution of fiber types is dependent on several variables including body length, mass, locomotory skills, and degree of maturity at hatching (Johnston and Horne 1994). Temperature strongly affects the size of larvae at hatching, and thus affects the fiber type distribution. For example, red sea bream (*Pagrus major*) larvae hatch at 17–23°C around 2.1 mm and contain a central core of white muscle fibers with a superficial layer of undifferentiated myoblasts (Matsuoka and Iwai 1984). These undifferentiated myoblasts begin to differentiate when the larvae reach 2.9 mm in length. In comparison, rainbow trout larvae that hatch at 4–10°C are composed exclusively of white fibers, and develop only superficial red muscle fibers at free-swimming stage around 50 days posthatch (Nag and Nursall 1972).

Muscle Development

Muscle development is dependent on the proliferation and differentiation of certain populations of stem cells. Specifically, skeletal muscle derives from somites, which form from the paraxial mesoderm. Mammalian myoblasts appear sequentially during development in different populations (somatic, embryonic, fetal, and adult), consistent with programming versus functional adaptation (Harris et al. 1989). Functional muscle development occurs in two main waves: (1) embryonic myoblasts lead to primary muscle fibers that form a scaffold and (2) the scaffold acts as a template for the formation of many secondary muscle fibers that develop from fetal myoblasts (Ontell et al. 1988; Wilson et al. 1992; Oksbjerg et al. 2004). In mammals and most terrestrial vertebrates, the definitive number of muscle fibers is preestablished in early development or shortly after birth (Rowe and Goldspink 1969; Schultz 1996). Following birth, growth occurs through the enlargement of existing muscle fibers by increases in length, diameter, and nuclear content (Rowe and Goldspink 1969; Schultz 1996). This type of growth is known as hypertrophic muscle growth, and occurs by the recruitment of cellular components to the enlarging fiber from a population of small myogenic progenitor cells, adult myoblasts, or satellite cells (Hawke and Garry 2001).

In teleosts, the axial musculature is made up of myotomes that are formed from repeating epithelial structures in the mesoderm (somites). Somites are derived from the paraxial mesoderm, located lateral to the notochord, and given rise to the schlerotome and myotome. In fish, the myotome is the major component of the somite, compared to mammals where the major component of the somite is the schlerotome. Also, muscle formation begins before all the somites of the trunk have formed and proceeds in a unidirectional manner, in a rostral to caudal direction. The myotome is separated into horizontal compartments by the horizontal myosepta. The dorsal compartment gives rise to the epaxial quadrants of the somite, while the ventral compartment gives rise to the hypaxial quadrant (Kronnie 2000).

Two compartments are developed from muscle precursors during embryogenesis in the segmental planes, resulting in different populations of cells giving rise to red and white fiber types (Devoto et al. 1996; Blagden et al. 1997). Adaxial cells migrate radially away from the notochord to form the superficial red layer, while lateral presomitic cells (muscle pioneer cells) remain deep in the myotome and form the thick layer of white muscle fibers (Kronnie 2000). Nonmigratory pioneer cells exhibit high levels of engrailed expression (Hatta et al. 1991), and subsequently elongate

the entire somite width to form the first muscle fibers (van Raamsdonk et al. 1978; Hanneman 1992). In zebrafish, adaxial slow muscle formation is under the control of sonic hedgehog (Shh) expression from the notochord (Blagden et al. 1997). Similarly, echidna hedgehog, a member of the Shh gene family, secreted from the notochord plays a role in the formation of pioneer cells that form the fast musculature (Currie and Ingham 1996). Interestingly, zebrafish that lack Shh expression fail to form slow muscle but exhibit unaffected fast muscle development, suggesting different signaling pathways that regulate the formation of fiber types (Blagden et al. 1997).

Molecular Regulation of Muscle Development

In higher vertebrates, the myogenic basic helix-loop-helix (bHLH) transcription factor gene family, collectively called myogenic regulatory factors (MRFs), have been extensively studied in relation to skeletal muscle growth. These transcription factors include MyoD, Myogenin, Myf5, MRF4 (Myf6), and MRF6, and play a central role in the determination and differentiation of skeletal muscle (Weintraub 1993). In mammals, overexpression, or forced expression, of each MRF can convert nonmuscle cells to myoblasts (Weintraub et al. 1991). However, individual knockout of these genes is not lethal, and muscle tissue development is normal, suggesting overlapping functional roles (Rudnicki et al. 1992; Smith et al. 1994).

MRFs form heterodimers with E-protein products, such as E-47 and E-12, and bind to the E-box DNA sequence motif (CANNTG) present in the regulatory region of many muscle-specific genes (Olson 1992). Upon dimerization, MRFs form a DNA-binding "pocket" allowing the complex to bind DNA. Id proteins, helix-loop-helix proteins without DNA-binding domains, can also bind MRFs, inhibiting their DNA-binding function (Cserjesi and Olson 1991; Lassar et al. 1991). Some growth factors produced in proliferating myoblasts stimulate Id expression to prevent myoblast proliferation and induce myotube fusion (Hasskarl and Munger 2002). The myocyte-specific enhancer factor 2 (MEF2) family of transcription factors controls the regulation of muscle cell differentiation by binding to A/T-rich sequences present in the promoter and enhancer site of many muscle-specific genes (Gossett et al. 1989).

Several MRFs have been cloned and characterized in fish, and expression has been demonstrated primarily by in situ hybridization specifically in developing somites and skeletal muscles. Rescan and coworkers first identified full-length MyoD in rainbow trout (Rescan et al. 1994) and later by using embryonic cDNA library screening techniques, they identified two MyoD isoforms (Rescan and Gauvry 1996). Because many fish exhibit significant muscle growth after embryogenesis, postlarval regulation of hyperplasia and hypertrophy are of research interest. As previously mentioned, hyperplastic growth in early ontogeny occurs in phases. The first phase is primary myotome formation, as described above. In teleost fish, this phase is subdivided into subphases: (1) myogenic precursor cells are determined (adaxial cells adjacent to notochord) and develop in a mediolateral migration to form the superficial slow fiber layer and (2) lateral paraxial cells within the newly formed somite give rise to the fast fiber layers (Devoto et al. 1996; Stoiber et al. 1998). The second phase of muscle growth involves the addition of new muscle cells at the myotome surface, generating "proliferation zones" at the dorsal and ventral extremities and the fast muscle layer boundary (Rowlerson and Veggetti 2001). This phase of growth is termed "stratified growth" as growth zones are generated in the apical region (Figure 11.5). The third

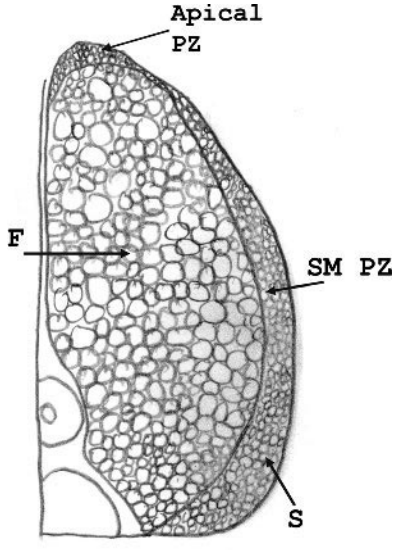

Figure 11.5. Schematic representation of epaxial lateral muscle growth of postlarval teleosts. The superficial monolayer proliferation zone (SM PZ) gives rise to slow muscle fibers in postlarval fish. The apical PZ gives rise to fast fibers and is responsible for stratified hyperplasia. (Adapted from Rowlerson et al. 1995.)

phase of growth is termed "mosaic growth" and involves fiber recruitment between preexisting muscle fibers scattered throughout the myotome, generating a mosaic-like appearance deep in the myotome (Figure 11.5). The first and second phases of muscle cell growth are well understood; however, the regulation of the fate determining pathways responsible for mosaic growth is still unclear. Figure 11.6 depicts the current understanding of cell progression and gene regulation in vertebrates.

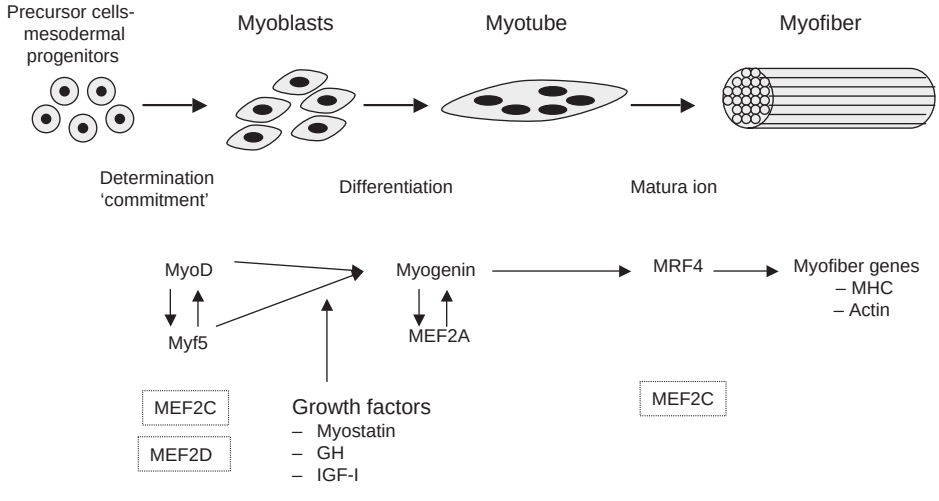

Figure 11.6. Schematic representation of muscle cell growth regulation. (Adapted from Olson and Klein 1994; Watabe 1999.)

Recent evidence suggests that fish species with a large final body size and rapid growth exhibit fast muscle mosaic growth early in development, immediately following adaxial cell migration (Steinbacher et al. 2007). These results suggest that the onset of the second and third phases of myogenesis occurs simultaneously and follows the first phase of growth, uninterrupted, in fish species characterized by indeterminate growth. Third-phase mosaic hyperplastic muscle growth is known to be responsible for the rapid increase in muscle mass seen in larvae and juvenile teleosts (Rowlerson and Veggetti 2001) and sustained growth in adult fish (Weatherley et al. 1988). Zebrafish, which grow only to a small final body size, exhibit slow-muscle-stratified hyperplasia following segmentation during embryonic development and this continues during the larval period (Barresi et al. 2001). Baressi and coworkers demonstrated that the mechanisms specifying these new slow fibers are different from those that identify adaxial-derived slow muscle fibers (Barresi et al. 2001). It is therefore hypothesized that different mechanisms have arisen to accommodate muscle growth phases and cell fate depending on species and environment.

Timing of MRFs coincides with overall regulatory function of the factor. Primary MRFs, such as MyoD and Myf5, play key roles in specific cell lineage determination, while secondary MRFs, such as myogenin and Myf6, are involved in regulating differentiation. In most of all vertebrate skeletal muscle lineage, the expression of Myf5 and MyoD is followed by the upregulation of Myogenin, Myf6, and MEF2 family of transcription factors (Yun and Wold 1996; Watabe 1999). In mice, however, Myogenin expression is detected prior to MyoD transcripts in precursor myotomal muscle cells (Sassoon et al. 1989). Overall, the expression of MyoD and Myf5 induces migration of newly committed myoblasts to the site of muscle formation (Rudnicki et al. 1992, 1993).

In nonamniotic vertebrates, such as fish, the expression of MyoD at the end of gastrulation induces cell migration to paraxial and axial mesoderm to form myogenic lineage cells in a row, known as adaxial cells (Watabe 2001). Myogenesis in amniotes is initiated much later, and the last MRF to be activated is MRF4, which is expressed into adult stages (Rhodes and Konieczny 1989). In fish, MyoD expression is found in adaxial cells of the presomitic mesoderm (Weinberg et al. 1996; Delalande and Rescan 1999; Temple et al. 2001; Xie et al. 2001; Tan and Du 2002; Hall et al. 2003; Cole et al. 2004; Zhang et al. 2006), demonstrating that mesodermal cells are destined to become muscle cells early in organogenesis. Steinbacher and coworkers demonstrated that in pearlfish (*Rutilus frisii meidingeri*), MyoD and MEF2D expressions are found simultaneously in adaxial slow fiber precursors and fast fiber precursors (Steinbacher et al. 2006). In brown trout, MyoD and myogenin expressions are maintained in areas of the myotome known to be stratified growth zones, suggesting that MyoD and myogenin transcripts are restricted to myoblasts and young myofibers of the proliferation zones (Steinbacher et al. 2007). In Atlantic halibut, MyoD expression is detectable prior to somitogenesis, and myogenin expression is first detected at the nine- to ten-somite stage (Galloway et al. 2006). Interestingly, MyoD transcripts were never detected in adaxial cells of the presomitic or somitic mesoderm in Atlantic halibut (Galloway et al. 2006) and Atlantic cod (Hall et al. 2003). These results suggest that MyoD has a different function in cod and halibut, and/or halibut and cod possess more than one MyoD gene as seen in several other fish species (Rescan and Gauvry 1996; Tan and Du 2002) and in *Xenopus* (Scales et al. 1990).

Trout express two nonallelic MyoD genes (TMyoD and TMyoD2) that exhibit differential spatial and temporal expression patterns during early development and postlarval stages (Delalande and Rescan 1999). Using digoxygenin-labeled probes and in situ hybridization, TMyoD expression was first detected in the presomitic mesoderm and somatic medial cells adjacent to the notochord (adaxial cells). In zebrafish, MyoD-expressing adaxial cells committed to a slow myoblast lineage is due to notochord-derived hedgehog (Hh) signaling (Weinberg et al. 1996; Blagden et al. 1997; Du et al. 1997). In comparison, TMyoD2 expression is not detected until somites have formed and expression is restricted to the posterior halves of the somites (Figures 11.7a and 11.7b). Following the completion of segmentation, expression of TMyoD expands laterally within the myotome (Figure 11.7c) and then TMyoD expression

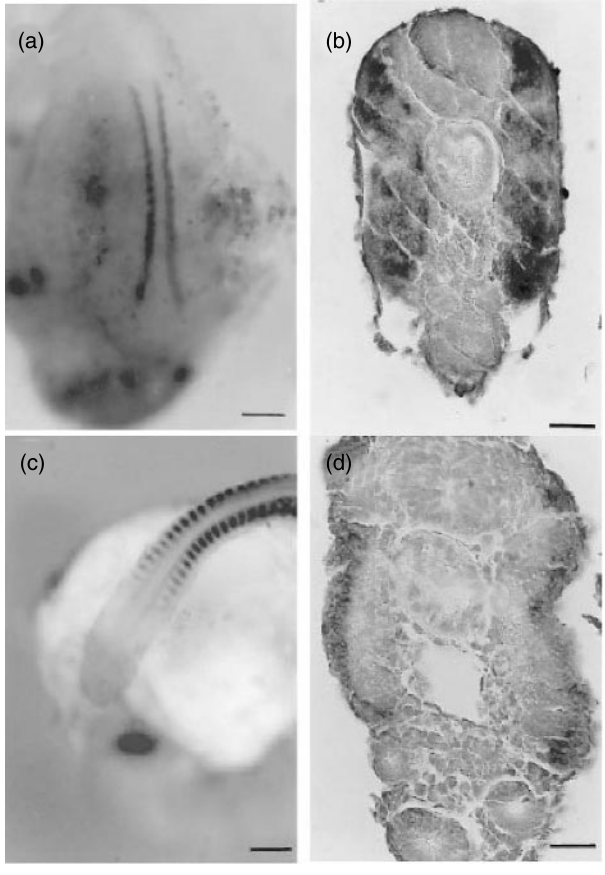

Figure 11.7. Expression of TMyoD (a, b) and TMyoD2 (c, d) in rainbow trout embryos. (b) Stage 11 embryo with approximately 20 somites. TMyoD expression is restricted to the adaxial cells of the somites. (c) Stage 14 embryos, TMyoD2 expression is restricted to the posterior domain of somites. (b, d) Stage 20, completion of segmentation. TMyoD expression undergoes lateral expansion (b), while TMyoD2 becomes confined to the peripheral domain of the myotome (d) (Delalande and Rescan 1999). (Reprinted with kind permission from Springer Science and Business Media.)

becomes confined to the peripheral domain of the myotome (Figure 11.7d) (Delalande and Rescan 1999). Interestingly, TMyoD and TMyoD2 are true orthologs of zebrafish MyoD based on sequence similarity, and complementary expression patterns of the two trout MyoD genes are similar to that of zebrafish MyoD suggesting that the two duplicated trout genes assume the role of the one ancestral gene (Delalande and Rescan 1999). When comparing MyoD expression across vertebrate taxa, it is important to point out that mammalian and bird MyoD expression only occurs following somite formation, while the initial expression of MyoD in lower vertebrates is first detected in the presomitogenic phase, in adaxial cells of fish and in the paraxial mesoderm of frogs. The functional importance of this discrepancy is still unclear.

Along with MyoD, Myf5 is required for complete myocyte and myofiber development. Myf5 has been identified in a few fish species, including the zebrafish (Chen et al. 2001), striped bass (Tan et al. 2002), rainbow trout (Johansen and Overturf 2005a), common carp (Kobiyama et al. 1998), sea perch (Ye et al. 2007), and flounder (Tan et al. 2006). Targeted gene knockdown with Myf5-morpholino results in myogenesis defects in zebrafish (Chen and Tsai 2002), similar to the lack of myoblast formation seen in MyoD and Myf5 gene knockout mice (Rudnicki et al. 1993). MRF5 and MyoD expressions in zebrafish are first seen at 7.5 days in bilateral bands of cells flanking the presumptive notochord (Weinberg et al. 1996; Chen et al. 2001; Coutelle et al. 2001). In *Xenopus*, Myf5 transcripts are detected in presomitic mesoderm prior to muscle differentiation (Hopwood et al. 1991). In zebrafish, Myf5 expression is detected in adaxial and lateral cells of the segmental plane and is only transient in developing somites (Chen et al. 2001). Both MyoD and Myf5 expressions in lower vertebrates appear before paraxial mesoderm segmentation. As shown by gene expression analysis, Myf5 is the first MRF-encoding gene to be expressed during embryogenesis in several vertebrates, including zebrafish, flounder, and common carp.

The expression patterns of MyoD and Myf5 overlap considerably in several vertebrates including zebrafish; however, in flounder the expression patterns are significantly different (Tan et al. 2006). As in most vertebrates, flounder Myf5 expression precedes MyoD expression, but in contrast Myf5 is expressed at high levels in lateral presomitic cells where MyoD expression is not detected (Tan et al. 2006). Also, Myf5 expression is limited to newly formed somites, while MyoD transcripts are found in all somites. In rainbow trout, Myf5 and TMyoD2 expressions exhibit different patterns: Myf5 expression increased from eyed-stage embryo to swim-up fry, but did not peak at swim-up like TMyoD2 expression (Johansen and Overturf 2005a). Sea perch Myf5 transcripts are also found early in gastrulation and throughout embryonic development, with high expression levels in the gastrula and somite stages (Ye et al. 2007). Unfortunately, the MyoD gene has not been characterized in the sea perch to date, so we can only speculate that different expression patterns between Myf5 and MyoD are exhibited. However, the early expression and continued high levels of Myf5 transcripts during development suggest similar patterns to trout. Due to variations demonstrated in teleosts, it is hypothesized that early myogenesis regulation plays an important role in muscle cell recruitment. Also, the regulatory pathways of MRFs are different between development and differentiation in determinate and indeterminate growing fish species.

Another important set of skeletal muscle differentiation factors includes the MEF2 family of transcription factors that bind A/T-rich sequences found in many muscle-specific promoters and enhancers (Gossett et al. 1989). MEF2 isoforms regulate

myogenic bHLH genes and in conjunction with MRFs regulate muscle-specific transcription (Olson 1992; Yun and Wold 1996). Three MEF2 genes have been described in zebrafish, two in carp (Ticho et al. 1996; Kobiyama et al. 1998), and two in rainbow trout (Johansen and Overturf 2005b). MEF2D is first detected in zebrafish at midgastrulation and is present in adaxial cells adjacent to the notochord in the presomitic mesoderm (Ticho et al. 1996), similar to MyoD (Weinberg et al. 1996). MEF2A and MEF2C expressions appear later, but follow similar somite expression patterns as MEF2D in a rostral to caudal sequence (Ticho et al. 1996). In carp, MEF2A expression is first detected in 15-somite stage embryos in concert with myogenin (Watabe 2001). Also, the MEF2C transcript expression precedes MEF2A and appears in concert with MyoD. In rainbow trout, MEF2A transcripts appear detectable at levels similar to myogenin and peak at very high levels at swim-up fry stage (Johansen and Overturf 2005a). In contrast, MEF2C transcript levels are comparatively low during development but extremely high in spawning females. In rainbow trout, however, the sequential activation of myogenic factors functioning downstream of MyoD is similar to that seen in zebrafish (Rescan et al. 1994, 1995), which do not exhibit any or little posthatch hyperplasia. However, developmental expression of MRFs, such as MEF2A and MEF2C, supports possible regulatory roles in muscle cell fate and recruitment.

Myogenin and MRF4 expressions are activated during terminal differentiation (Rhodes and Konieczny 1989; Wright et al. 1989; Edmondson and Olson 1993; Pownall et al. 2002). Knocking out myogenin expression in mice results in the formation of myoblasts, but these fail to fuse into muscle fibers (Hasty et al. 1993; Venuti et al. 1995). Also, these mice lacking myogenin functionality die at birth due to severe muscular deficiency, despite normal MyoD and Myf5 levels (Hasty et al. 1993; Nabeshima et al. 1993). Unique myogenin homologs have been identified in trout and zebrafish, and are found to be each derived from a unique myogenin gene (Weinberg et al. 1996; Delalande and Rescan 1999; Rescan et al. 1999). In Atlantic halibut, myogenin transcripts were first detected at the 9- to 10-somite stage in adaxial cells, and then spread to the lateral somatic cells of newly formed somites (Galloway et al. 2006). Myogenin signals disappear from cranial somites around the 30- to 40-somite stage, resembling myogenin expression patterns in Atlantic herring and common carp (Temple et al. 2001; Cole et al. 2004), but different from that seen in rainbow trout and zebrafish (Weinberg et al. 1996; Delalande and Rescan 1999). Interestingly, only one myogenin gene has been identified in Salmonids. It is unclear at this point whether there is another gene, different isoforms, or if the other gene was lost during evolution. It is important to investigate the possibility of multiple myogenin genes to fully characterize its function in fish.

MRF4 exhibits biphasic expression in mammals, with expression primarily concentrated in terminally differentiated myotubes and is not detected in mouse embryo skeletal muscle (Bober et al. 1991; Hinterberger et al. 1991). MyoD and MRF4 appear to have some overlapping functions, as either is required for differentiation alongside myogenin (Tajbakhsh and Buckingham 2000). In adult muscle, MRF4 expression predominates the MRF transcript expression and appears to be differentially regulated between different fiber types (Miner and Wold 1990; Walters et al. 2000; Pin and Konieczny 2002). In zebrafish, MRF4 expression is absent from presomitic adaxial cells, accumulates around the 13-somite stage in terminally differentiated slow fibers, and parallels myofibrillar myosin protein assembly (Hinits et al. 2007). Interestingly, MRF4 expression was not detected in presomitic mesoderm even though

terminally differentiated adaxial cells are present. Little information is available for MRF4 expression in teleosts, but is assumed to play an important role in terminal differentiation. It is likely that teleosts exhibiting indeterminate growth will have different MRF4 expression patterns congruent with other MRFs; MRF4 likely plays an important role in cell fate.

Muscle Cell Growth Regulation

The proliferation, differentiation, and growth of muscle in postlarval vertebrates are regulated by various factors, including endocrine, autocrine, and paracrine. Many of these factors influence the progression or regression of the cell cycle directly, but can also function indirectly through enzymes and transcription factors. Cyclin-dependent kinases (Cdk) are enzyme catalysts for events of the cell cycle, and their regulation establishes a link between myoblast cell cycle withdrawal and differentiation (Kitzmann and Fernandez 2001). During myogenesis, myoblasts proliferate and withdraw from the cell cycle at G_1, the first gap phase (Olson 1992). Commitment to the myotube differentiation pathway is established in G_1 and is coupled with the induction of MRF4 for the permanent withdrawal from the cell cycle to become terminally differentiated myotubes. G_1 cylin-Cdk, cyclin-D-Cdk, and cyclin-E-Cdk2 complexes cooperate to control the G_1 to S-phase transition through the phosphorylation and inactivation of retinoblastoma (Rb) protein (Guo and Walsh 1997). Two families of cyclin-dependent kinase inhibitors (CKI), p16 and p21, regulate the activity of Cdk. The family of p16 inhibitors specifically regulates Cdk4 and Cdk6, while p21 inhibitors act as a transition stage between G_1- and S-phases (Sherr and Roberts 1999).

The transforming growth factor-β (TGF-β) superfamily of genes encode secreted factors that are known to regulate embryonic development, and some TGF-β family members activate CKI, such as p21 (Kingsley 1994). Myostatin (MSTN), a new member of the TGF-β superfamily expressed in developing and adult muscle tissue (McPherron et al. 1997), controls myoblast cell cycle progression. Using cultured C_2C_{12} myoblast cells and a recombinant MSTN protein, Thomas and coworkers demonstrated that MSTN acts negatively to regulate muscle cell progression by increasing p21 and decreasing Cdk2 protein and activity levels (Thomas et al. 2000). The downregulation of Cdk2 renders the cyclin-E-Cdk2 complex inactive, resulting in decreased phosphorylation of Rb and subsequent regulation of the cell cycle at the G_1- to S-phase and G_2- to M-phase transitions (Thomas et al. 2000). Cell cycle arrest occurs due to the role dephosphorylated Rb has on inactivating gene transcription regulatory proteins (Sherr and Roberts 1999). The removal of MSTN expression results in increased cell proliferation, and both hyperplastic and hypertrophic muscle growth (Thomas et al. 2000).

Several MSTN genes have been isolated and characterized from many fish species (Maccatrozzo et al. 2001a, 2001b; Ostbye et al. 2001; Rescan et al. 2001b; Roberts and Goetz 2001; Rodgers and Weber 2001; Rodgers et al. 2001; Kocabas et al. 2002; Xu et al. 2003; Biga et al. 2005; Kerr et al. 2005). Recent phylogenetic analysis of the MSTN subfamily (Kerr et al. 2005) indicates that bony fish genomes encode for two MSTN genes (*MSTN1* and *MSTN2*) due to an early gene duplication event (Amores et al. 1998; Postlethwait et al. 1998). A second duplication event in salmonids, presumably resulting from tetraploidization, produced two subsequent divisions

within each MSTN clade, resulting in two isoforms from each MSTN gene (*MSTN1a* and *1b*, and *MSTN2a* and *2b*) (Garikipati et al. 2006; Garikipati et al. 2007). Mammals encode one MSTN gene whose expression is limited predominately to skeletal muscle (McPherron et al. 1997), to a lesser extent in heart (Sharma et al. 1999), and little expression in mammary (Ji et al. 1998) and placental tissue (Mitchell et al. 2006). In fish, MSTN expression is detectable in many different tissues (Ostbye et al. 2001; Rodgers et al. 2001; Radaelli et al. 2003; Roberts and Goetz 2003; Amali et al. 2004; Kerr et al. 2005) and is differentially regulated (Rescan et al. 2001b; Roberts and Goetz 2003; Biga et al. 2004). Tissue-specific functions of MSTN have yet to be determined, and it is truly speculative that MSTN negatively regulates muscle growth in fish species at this point based on the mammalian literature.

MSTN is transiently expressed during development, and is transcriptionally regulated (Kambadur et al. 1997; McPherron et al. 1997). MSTN is first expressed in myogenic precursor cells of the myotome compartment of developing somites, and then in adult axial and paraxial muscles (McPherron et al. 1997). Differential expression is detected between different axial and paraxial muscles (Kambadur et al. 1997). In developing rainbow trout, MSTN1a and 1b are expressed in similar patterns, with low expression throughout development (Biga et al. 2005; Johansen and Overturf 2005a; Garikipati et al. 2006). MSTN expression increases around eyed-stage, and MSTN1a levels are consistently higher than MSTN1b levels (Garikipati et al. 2006). Brook trout MSTN expression is similar with low levels detectable by qRT-PCR, and levels increase around eyed-stage and continue increasing through development (Roberts and Goetz 2003). Very low levels of MSTN expression are detectable in zebrafish embryos, as demonstrated by failed in situ hybridization and successful RT-PCR reactions (Xu et al. 2003). Xu and coworkers could not detect MSNT expression in zebrafish embryos with whole-mount in situ hybridization until 4 days postfertilization.

The promoter/enhancer region of the human MSTN gene contains response elements for glucocorticoids, androgens, thyroid hormones, myogenic differentiation factor 1, myocyte enhancer factor 2, peroxisome proliferator-activated receptor, and nuclear factor-κB (Ma et al. 2001). Cloning of the bovine MSTN promoter revealed that it contains ten E-box motifs (CANNTG) and a single MEF2 site (Spiller et al. 2002). Analysis of murine MSTN promoter region revealed the presence of seven E-box motifs (Salerno et al. 2004). E-boxes are the binding sites for MRFs (Murre et al. 1989; Lassar et al. 1991), including MyoD, Myf5, myogenin, and MRF4 (Hinterberger et al. 1991; Rudnicki et al. 1992; Rudnicki et al. 1993). MyoD binds to the E6 E-box motif in vitro and in vivo (Spiller et al. 2002), and high MSTN promoter activity was detected in concert with high levels of MyoD expression during the G_1-phase myoblast cell cycle (Kitzmann et al. 1998). Also, Salerno and coworkers demonstrated preferential activation of the MSTN promoter by MyoD (Salerno et al. 2004), suggesting that MyoD regulates the MSTN gene and thus cell cycle withdrawal.

Sequence analyses of fish MSTN promoter regions have identified several *cis*-regulatory elements (Roberts and Goetz 2003; Xu et al. 2003; Garikipati et al. 2006), which might contribute to myogenesis regulation. In the zebrafish, several E-box motifs were identified, with two closely linked E-boxes (E5 and E6) present in a similar position to those in the bovine MSTN promoter (Xu et al. 2003). In brook trout, the MSTN gene promoter regions contain two MEF2 sites, two E-boxes, a growth hormone cell-specific element (GH-CSE2), AP1, AP2, SP1, cAMP response

element, and CCAAT box sites (Roberts and Goetz 2003). Isoform-specific promoter elements were identified, including an NF-κB site, androgen response element and glucocorticoid response element in btMSTN1b, and an SF1 site in btMSTN1a. Rainbow trout MSTN1a and 1b contain several of these cis-regulatory elements including E-boxes and TATA boxes, as well as SRF (serum response factor) and TEF-1 (transcription enhancer factor 1) binding sites (Garikipati et al. 2006). A MusIn (muscle initiator) binding site was also identified in rtMSTN1a and a MEF3 site in rtMSTN1b. The rtMSTN2 isoforms also contain MEF2, SRF, TEF-1, and COMP1 binding sites, as well as several putative E-boxes and TATA boxes in the promotor region (Garikipati et al. 2007). In addition, a MyoD-binding site is present in the rtMSTN2a promoter. Expression of fish MSTN increases with somitogenesis and rapidly decreases as it ends, consistent with MRF expression seen in all vertebrates and MSTN expression patterns seen in developing mice (McPherron et al. 1997).

It is still unclear, however, if MSTN functions in fish as seen in mammals. Transgenic zebrafish expressing the MSTN prodomain were generated to investigate the function of MSTN in zebrafish muscle growth (Xu et al. 2003). A gene encoding the zebrafish MSTN prodomain was linked with the rat myosin light chain gene promoter/enhancer, and the resulting construct was microinjected into zebrafish embryos for the production of transgenic fish. Whole-mount in situ hybridization was used to detect the MSTN prodomain transgene, demonstrating that the transgene was strongly expressed in developing somites and embryonic muscle in transgenic fish (Xu et al. 2003). Further analyses demonstrated no significant changes in morphology, growth, development, or expression of MRFs, suggesting that MSTN does not play an important role in myogenesis in early-stage embryos. In contrast, the knockdown of MSTN expression by an antisense morpholino resulted in enhanced expression of MRFs (Amali et al. 2004). This is consistent with overexpression of MSTN in C_2C_{12} cells resulting in the inhibition of MRFs (Langley et al. 2002; Rios et al. 2002; Joulia et al. 2003). Adult transgenic fish exhibited approximately only 10% more myofibers than nontransgenic controls with no difference in fiber size, indicating a slight increase in hyperplasia (Xu et al. 2003). These results suggest that MSTN might play a negative regulatory role in hyperplastic muscle formation, but no effect on hypertrophy. These results, however, are not truly representative of muscle growth regulation in fish species. The increased hyperplasia demonstrated by Xu and coworkers was stratified hyperplasia, which all fish species exhibit. However, mosaic hyperplasia is almost entirely lacking in postlarval zebrafish (van Raamsdonk et al. 1983; Weatherley and Gill 1984; Weatherley et al. 1988), and it is hypothesized that large fish species important in aquaculture would exhibit dramatic muscle growth differences in both hyperplasia and hypertrophy (van Raamsdonk et al. 1983; Weatherley and Gill 1984; Weatherley et al. 1988).

Differential growth paradigms were recently demonstrated between two closely related teleosts, the zebrafish and giant danio (Biga and Goetz 2006). Morphometric analysis of giant danio and zebrafish larvae demonstrated faster, more efficient growth in giant danio larvae. Total myotome area, mean fiber area, and total number of fibers all exhibited positive correlations with larval length in only the giant danio, suggesting that giant danio exhibit indeterminate growth with significant postlarval hyperplasia. A progressive increase in mean fiber area in zebrafish larvae demonstrates predominately hypertrophic postnatal growth, which is consistent with other small fish species where hypertrophy is the main growth type (Weatherley and Gill 1984). Given the

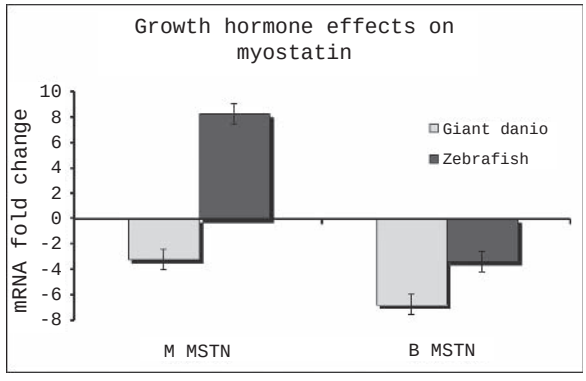

Figure 11.8. Gene expression fold change in growth hormone treated versus nontreated control zebrafish and giant danio. Relative myostatin expression in muscle and brain (Biga and Goetz 2006). Myostatin expression is repressed in response to GH treatment in giant danio, an indeterminate growing species. In contrast, GH increases muscle MSTN in zebrafish, a determinate growing species.

muscle growth dynamics of the giant danio, it provides an important representation of many commercially relevant fish species. More recently, it was demonstrated that exogenous growth-promoting agents have differential effects on MSTN expression between zebrafish and giant danio (Figure 11.8; Biga and Goetz 2006). Myostatin appears to be suppressed in muscle and brain tissue following growth hormone treatment in the giant danio, which exhibits indeterminate growth. In contrast, growth hormone treatment increases MSTN expression in muscle tissue of the determinate growing zebrafish. These results are consistent with previous evidence that growth hormone has little to no growth-promoting effects in zebrafish (Morales et al. 2001; Biga and Goetz 2006), and it appears that MSTN might be an important regulator of hyperplastic growth. More research is needed on the functional characterization of MSTN in relation to muscle growth and developmental regulation in fish, with particular interest paid to species or models relevant to aquaculture production.

Adult Myoblast Growth Regulation—*Satellite Cells*

Quiescent muscle precursor cells located in the basal lamina of the muscle fiber, called satellite cells, are stem cell-like cells. In 1961, Mauro described these cells as satellite cells based on their location, which is distinct from the myofiber between the basal lamina and sarcolemma (Mauro 1961). These satellite cells are responsible for the adaptable nature of skeletal muscle in terms of growth, fiber switching, training, and trauma response. Satellite cells appear in the limbs of mammals at approximately 17.5 days postcoitum, following primary muscle fiber formation. Satellite cells are thought to originate from a pool of precursor cells from the dermomyotome cell population (Gros et al. 2005), but there is evidence that they might also originate from the dorsal aorta (Seale and Rudnicki 2000). Much attention has been paid to investigate the function and regulation of mammalian satellite cells, as they are important in muscle repair and regeneration, and less attention has been paid to satellite cells

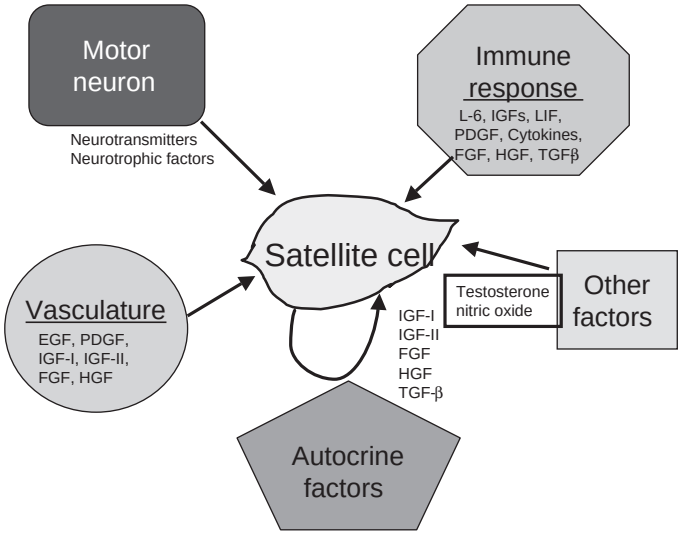

Figure 11.9. Model for satellite cell activation. (Modified from Hawke and Garry 2001.)

from fish species. As described before, myogenesis continues posthatch in several fish species, so satellite cells will account for both hypertrophic and hyperplastic growth. It is difficult to demonstrate satellite cell involvement in these processes in fish, but the involvement should be considered more dynamic than what is known in mammals.

Mammalian satellite cells are known to contribute to muscle growth by hypertrophy of existing fibers and to muscle regeneration by rebuilding damaged fibers (Stockdale 1992). In several fish, satellite cells have the ability to fuse with existing myofibers or differentiate into new myofibers (Fauconneau and Paboeuf 2001a). Satellite cells, also termed adult skeletal "stem cells," are a renewable source of cells utilized for muscle repair and regeneration (Chen and Goldhamer 2003). In mammals, the physiological context of satellite cell activation is known (i.e., following acute injury or exercise) and a moderate understanding of the molecular and/or hormonal factors that are involved in the initiation of satellite cell proliferation is understood (Figure 11.9).

Until recently, electron microscopy was the only method of accurate satellite cell visualization. Hawke and Garry (2001) demonstrated that satellite cells express specific proteins that are not expressed in postmitotic myofibers, including M-cadherin, c-met, hepatocyte growth factor receptor, myocyte nuclear factor, and Pax7. Utilizing immunohistochemical and immunocytochemical techniques, satellite cell visualization is much easier and accurate.

Fish satellite cells have been characterized in situ (Nag and Nursall 1972; Willemse and van den Berg 1978; Koumans and Akster 1995b; Stoiber and Sanger 1996) as small cells (>5 μm), containing a heterochromatic nuclei and reduced cytoplasm. In mammals, satellite cells are typically uniformly distributed longitudinally and lie near myofiber nuclei. In fish, they are located at multiple points along myofibers and more specifically at an angle to the polygonal shape (Willemse and van den Berg 1978; Koumans et al. 1991).

The isolation and in vitro propagation of satellite cells in mammals have resulted in the identification of satellite cell-specific markers (see above) as well as local activation and regulation. Attempts to develop fish muscle cell lines have fallen short of success (Hightower and Renfro 1988). However, the development and utilization of primary myoblast cultures have been successful in some fish species, including trout (Powell et al. 1989; Greenlee et al. 1995a, 1995b; Castillo et al. 2002), salmon (Matschak and Stickland 1995), carp (Koumans et al. 1990), zebrafish (Sepich et al. 1994), and giant danio (Biga and Goetz 2006). Fauconneau and Paboeuf (2001a) detail the various methods of myoblast culture, so this chapter does not duplicate the information. To date, few studies have been conducted using primary myoblast cultures from fish. Investigations on metabolic receptor function (Castillo et al. 2002, 2004, 2006), nutritional physiology (Fauconneau and Paboeuf 2000), and toxicology studies (Fauconneau and Paboeuf 2001b) have utilized this methodology. However, no report has characterized the primary myoblast cultures or evaluated the molecular and hormonal regulation involved in vitro. An important future direction for this area of research in fish muscle growth needs to focus on the characterization of the myoblast populations present.

In mammals, satellite cells are activated by trauma or injury. It is hypothesized that similar mechanisms are present in fish; however, activation outside of mechanical response must be present to accommodate the continuous growth exhibited by many fish species. In mammals, following injury or trauma, *Pax-7*- and *FoxkI*-positive satellite cells are activated to proliferate, resulting in reentry into the cell cycle to form proliferating myogenic precursor cells expressing MRFs, *Myf5* and *MyoD* (Eftimie et al. 1991; Buonanno et al. 1992; Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997; Beauchamp et al. 2000), the myoblast marker *desmin* (Allen et al. 1991; Cornelison and Wold 1997), the cell surface marker M-cadherin (Irintchev et al. 1994; Hollnagel et al. 2002), and *Wnts 5a* and *5b* (Polesskaya et al. 2003). The *Notch* signaling pathway regulates this activation (Conboy and Rando 2002), and several growth factors, such as insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (FGF), and hepatocyte growth factor/scatter factor (HGF/SF), stimulate/regulate myoblast proliferation (Allen and Boxhorn 1989; Lefaucheur and Sebille 1995; Tatsumi et al. 1998). As *Pax-7* expression declines, the expression of *MRF4* and *Myogenin* is upregulated (Eftimie et al. 1991; Buonanno et al. 1992; Cornelison and Wold 1997), and differentiated myoblasts fuse to fibers for repair. Interestingly, bone marrow-derived stem cells can contribute directly to this process by becoming satellite cells and subsequently differentiating into multinucleated myofibers following injury or exercise-induced trauma (LaBarge and Blau 2002). However, why the repair and regeneration mechanisms involving satellite cell populations are not active or available during cachectic states (sarcopenia) and whether fish satellite cells act in a similar manner is not known.

Muscle Growth

As previously discussed, an interesting difference between most fish species and mammalian vertebrates is that many fish have the ability to recruit new fibers to form muscle (hyperplasia) during adult growth. Both hyperplasia and hypertrophy (enlargement of preexisting fibers) occur during myogenesis in larval and adult fish that obtain a

large adult size (Stickland 1983; Weatherley et al. 1988; Koumans and Akster 1995b; Alami-Durante et al. 1997; Patruno et al. 1998). In mammalian normal growth, myotube formation ceases after birth and hypertrophy predominates growth throughout life (Rowe and Goldspink 1969). However, various stimuli, including exercise (Darr and Schultz 1987) and injury (Snow 1977; Blaveri et al. 1999), can trigger the formation of new myotubes. The extent of satellite cell participation is unknown in fish, and the degree of contribution of hyperplasia and/or hypertrophy is dependent on both the species and the final total body size of the fish. Fish species exhibiting a smaller somatic size generally exhibit hyperplastic growth early only in development and rely on hypertrophic growth later in development. Conversely, fish that attain larger overall body size appear to recruit new muscle fibers throughout adulthood, which suggests that hyperplasia might be the determining factor in overall body size of fishes.

When assessing the contributions to muscle growth by either hypertrophy or hyperplasia, measuring muscle fiber diameters, or cross-sectional areas, provides a good quantitative method for understanding the mechanisms of somatic growth in fish. Also, extent of satellite cell activation can be an important tool in evaluating growth and possibly hyperplastic growth involvement. Because growth is dependent on age, size, and environmental condition, it is imperative to take these factors into account. Many studies since the early 1960s have provided the basis for our current understanding of how muscle fiber growth and recruitment is species and temperature dependent. The distribution of fiber areas in a given myotomal section is used as a measure of the appearance of newly developed fibers, or an indicative measure of hyperplasia. The presence of very small diameter fibers ($<$20 μm) is considered indicative of hyperplastic growth, but not an indicator of rapid growth as they are related to fish size rather than growth rate (Weatherley and Gill 1982, 1987; Weatherley et al. 1988).

The total number of muscle fibers in a consistent cross-sectional myotomal region, such as a transverse section of the trunk, is also utilized as a tool to understand somatic growth. Sometimes fiber diameter and number are combined to calculate cellularity (fiber number in relation to fiber area). The relation of nuclear numbers to fiber areas or total numbers can also provide useful information about overall growth in fish. This information is reviewed in depth by Rowlerson and Veggetti (2001) and is not within the scope of this book, but imperative to our understanding of somatic growth regulation in fishes.

Evidence suggests that in finfish that reach a large final body size achieve this by early somatic growth via hyperplastic growth, and this hyperplasia decreases for a given species as the size increases (Weatherley et al. 1980; Stickland 1983; Weatherley and Gill 1984, 1985; Koumans et al. 1993). In adult, juvenile, and larval carp, the percentage of growth accounted for by hyperplasia decreased from 66% at $>$3 cm of fish length to 30% at 5 cm of fish length, and then to 0% at 34 cm of fish length (Koumans et al. 1993). Also, decreased hyperplastic growth contributions appear to be related to slow-growing phenotypes of some carp strains (Alami-Durante et al. 1997). In contrast, rainbow trout recruitment of new fibers is directly related to growth rate in small juveniles (Weatherley and Gill 1981), but it appears that hyperplasia is favored during slow growth while fast growth favors hypertrophy in posthatching trout (Kiessling et al. 1991). Consistent with carp, juvenile salmon exhibit increased hyperplasia during fast growth and hypertrophy is more important during slow growth (Higgins and Thorpe 1990). In sea bream, hyperplasia continues postlarvally, but is

contained to the superficial monolayer and gives rise to slow fibers only (Rowlerson et al. 1995). And consistent with most other finfish, juvenile and adult sea bream growth occurs predominately by hypertrophy (Rowlerson et al. 1995).

Morphometric analysis of giant danio and zebrafish larvae demonstrated faster, more efficient growth in giant danio larvae (Biga and Goetz 2006). Total myotome area, mean fiber area, and total number of fibers, all exhibited positive correlations with larval length in only the giant danio, suggesting that giant danio exhibits indeterminate growth. A progressive increase in mean fiber area in zebrafish larvae demonstrates predominately hypertrophic postnatal growth, which is consistent with other small fish species where hypertrophy is the main growth type (Weatherley and Gill 1984).

In fish species, stratified hyperplasia is thought to be a continuation of embryonic myogenesis into larval development, as it occurs in the proliferation zones just below the superficial monolayer (Rowlerson and Veggetti 2001). Growth in the proliferation zones completes the formation of the distinct layers of red, pink, and white fibers that is started during embryonic development. Mosaic hyperplasia is responsible for large increases in the number of fibers in all layers of muscle, particularly in the fast-white layer (Rowlerson and Veggetti 2001). Interestingly, giant danio exhibits both stratified and mosaic hyperplasia in adult fish, while zebrafish adults exhibit only a degree of stratified hyperplasia at early larval stages (Biga and Goetz 2006). This is consistent with previous reports showing the reduction or lack of mosaic hyperplasia in zebrafish and other small fish species (van Raamsdonk et al. 1983; Veggetti et al. 1993; Koumans and Akster 1995a). Given these results, these two closely related species exhibiting opposing growth paradigms represent a powerful comparative growth model system.

Molecular Regulation of Myosin

The most abundant protein in muscle is myosin, and myosin serves as both a structural and a contractile unit. The myosin molecules of the myofibril are made up of six polypeptide chains, consisting of two heavy and four pairs of light chains. The two heavy chains are supercoiled as α-helices over predominately all the length, with a globular N-terminal head. The light chain pairs are broken apart in essential and regulatory light chains, both acting as calcium-binding proteins. Myosin proteins exist in many isoforms encoded in a family of different genes, where a minimum of three genes must be transcribed to make a single myosin molecule. The three genes must include one for the heavy chain, one for the essential light chain, and one for the regulatory light chain.

Twelve distinct genes of myosin protein classes have been described (Sellers et al. 1996), and only one, myosin class II, is involved in muscle contraction. In vertebrate muscle, myosin heavy chain (hc) genes encoding the molecular motors exist as a family of individual genes, but encode for different isoforms distinctly expressed during mammalian development or specifically to a fiber type (Weiss and Leinwand 1996). These isoforms include embryonic, neonatal, extraocular, cardiac-α and -β, and adult fast 2A, 2B, and 2X. Cardiac-β myosin hc isoform is expressed in both cardiac muscle and slow type I fibers. Therefore, in mammals, myosin hc expression is utilized as a main marker for muscle fiber phenotype during growth and development.

Fish express different myosin hc proteins as well, and studies have shown greater diversity in the myosin hc gene in fish than in mammals. There are 10 different class II myosin hc genes expressed in mammalian skeletal muscle, but tetraploid fish appear to express more than 10. Gerlach and coworkers presented evidence for 28 different myosin hc genes in carp (*Cyprinus carpio*) (Gerlach et al. 1990). Extensive sequence homology exists between species and is most highly conserved in the globular head region (Karn et al. 1983; Strehler et al. 1986). Interestingly, all vertebrate myosin hc genes exhibit two common features: (1) 40 28-residue repeats, with 39 complete as the 40th repeat contains the termination of the rod region, and (2) an extra "skip" residue with an unknown function at the end of 4-residue repeats (Stedman et al. 1990).

A complex shift in myosin hc expression occurs during the development of muscle fibers. In terrestrial vertebrates, primary muscle fibers express embryonic myosin hc at high levels compared to lower levels of neonatal and slow type I isoforms (Dhoot 1986; Narusawa et al. 1987). Secondary fibers, formed from the fusion of primary fibers, express only neonatal myosin hc isoform, and shortly after birth the neonatal myosin hc isoform disappears. As the expression levels of the developmental isoforms decrease and the fast and slow myosin hc isoforms begin to be expressed, the muscle fibers begin to develop phenotypic characteristics (Barbet et al. 1991; Pedrosa-Domellof and Thornell 1994). Developing muscles are considered slow, as the maximum shortening time is identical in fast and slow muscles, and become fully differentiated around 10 days after birth (Close 1964).

Most anatomical groups of muscles in terrestrial vertebrates are made up of a mosaic of different fiber types, exhibiting differing contractile properties and mATPase activities and stabilities (Figure 11.2). In comparison, slow and fast muscle fibers of fish species form physically discrete layers with slow fibers residing subcutaneously around the horizontal septum and fast fibers constituting the majority of the myotomal musculature (van Raamsdonk et al. 1982). Recent work has demonstrated a lateral migration of slow myosin hc-expressing cells from the medial to the lateral domain of the somite (Rescan et al. 2001a). Interestingly, this is in contrast to muscle differentiation as described in the zebrafish, which migrate laterally prior to the end of somitogenesis. It was recently demonstrated that adult zebrafish, which appear to exhibit determinate growth patterning, exhibit varying mATPase stability compared to a close relative exhibiting indeterminate growth patterning, the giant danio (*Danio aequipinnatus*) (Biga and Goetz 2006). Figure 11.3 demonstrates this difference in stability between the two fish and suggests that fish adaptation could play an important role in fiber-type characteristics. The important role that myosin hc expression has played in understanding fish myotomal development is discussed later in the chapter.

Histochemical and immunohistochemical analyses implicated that a different myosin hc isoform was expressed in small-diameter fibers in the white myotomal region in carp (Rowlerson et al. 1985). Utilizing a carp genomic library and previously identified different lambda clones containing myosin hc gene sequences, a novel myosin hc gene was isolated from small-diameter muscle fibers in carp, the FG2 myosin hc gene (Ennion et al. 1995). Interestingly, the FG2 myosin gene was not expressed in slow or large fast muscle fibers. Taking advantage of the unique FG2 untranslated region (UTR) as a probe, in situ hybridization revealed that FG2 expression is restricted to only small-diameter white fibers in fast-growing carp (Ennion et al. 1995) (Figure 11.10). Small-diameter fibers (<25 μm diameter) present

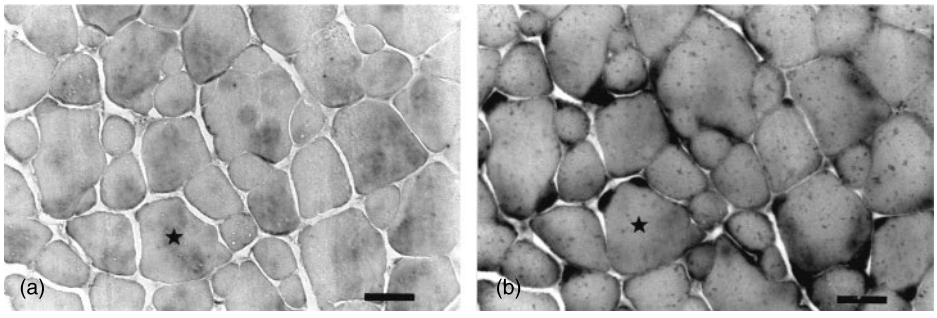

Figure 11.10. In situ hybridization using digoxygenin-labeled FG2UTR probe on carp muscle. The star indicates the same muscle fiber in serial sections. (a) Sense-labeled FG2UTR probe and (b) antisense-labeled FG2UTR probe. Scale bars, 50 μm. (Reproduced from Ennion et al. 1995 with permission.)

in the fast-white fiber myotome of adult fish are assumed to be myosatellite cells or derivatives of satellite cells via hyperplasia. Ennion et al. (1995) showed that FG2 is expressed transiently during the differentiation of satellite cells into muscle fibers, suggesting a role of a specific myosin hc in muscle cell recruitment. Various embryonic and neonatal myosin hc genes are expressed in differentiating mammalian satellite cells following injury or trauma (Sartore et al. 1982). However, carp myosin FG2 is not expressed in juvenile, fry, or developing embryos of carp, demonstrating that it is not an embryonic or neonatal isoform, but a more specific "growth isoform gene." An equivalent growth isoform has not been identified in any terrestrial vertebrate, which highlights the unique growth attributes seen in fish. However, a similar unique growth isoform of myosin hc has not been identified to date in other fish species either. The phenomena of continual fiber recruitment in adult fish are covered in detail later in the chapter.

One dynamic property of muscle tissue is the extent of plasticity it exhibits. Both skeletal and cardiac muscles adapt their mass and contractile protein isoforms to accommodate modifications to functional requirements. For example, when a slow muscle (e.g., soleus) is stimulated at high frequency, the muscle responds by shortening faster and acquires the fast myosin hc isoform. In skeletal muscle, the slow-to-fast muscle transition is accomplished by denervation, while the fast-to-slow muscle shift is accomplished by endogenous programming to yield an improved efficiency (see review, Swynghedauw 1986). In fish species, fiber-type switching probably is not as important as it is in terrestrial vertebrates, as the whole myotome in fish is designed to accommodate sustained and burst swimming activity. In fact, the chevron-shaped myotomal arrangement is associated with the need to accommodate constant sarcomere shortening at different body flexures (Alexander 1969; van Raamsdonk et al. 1980; Rome and Sosnicki 1990).

Temperature acclimation plays an important role in mATPase activity in fish that have evolved to live at vastly different temperatures. Fish muscle ATPase can adapt to seasonal environmental temperature variations by rebuilding their myofibrillar system for warm or cold water temperature (Johnston et al. 1973; Davison 1997). Specific ATPase activity is higher in colder temperatures, but exhibits less thermostability

than at more tropical temperatures. As previously mentioned, endothermic fish like tunas exhibit the ability to retain heat in the slow musculature that enhances muscle performance. Different species have different tolerances to varied environmental temperature depending on their ability to maintain body temperature or adapt to external temperatures. Therefore, it is hypothesized that different mechanisms regulate adaptation versus thermal maintenance of the musculature in different species. Unfortunately, research is limited to a few fish species, namely, zebrafish and carp, in regard to molecular regulation of myosin adaptation.

The first report of temperature acclimation through mATPase activity came from Davison (1997) when he acclimated goldfish (*Carassius auratus*) to either cold (10°C) or warm (26°C) water. He demonstrated that mATPase activity was around 2.8 times greater in cold-acclimated fish. Later, Heap et al. (1985) demonstrated similar changes in mATPase activity in other cyprinds including carp (*Cyprinus carpio*), tench (*Tinca tinca*), and roach (*Rutilus rutilus*). This report also demonstrated that a different regulatory system and genes control the acclimation of mATPase activity between temperature and starvation (Heap et al. 1985, 1986).

The role of mATPase in adaptation of phenotypic function is important for thermodynamic and mechanochemical efficiency. More precisely, the specific myofibrillar ATPase determines the rate of energy usage by the contractile units. Several early reports suggested that changes observed in myofibrillar ATPase proteins during temperature acclimation were due to the production of different contractile protein isoforms instead of the rearrangement of existing proteins. In carp, chymotrypsin digestion of myosin hc from warm- (30°C) to cold- (10°C) acclimated muscle resulted in differences in peptide maps (Hwang et al. 1990). The thermal stability of the myosin hc was demonstrated to be different in thermal acclimation, as the inactivation coefficient Ca^{2+}-dependent myosin was four times greater in cold- (10°C) versus warm- (30°C) acclimated carp (Hwang et al. 1990).

To gain better insight into how fish exhibit such plasticity and adaptability in muscle and metabolism, a more in-depth comparative analysis is needed across and within species that exhibit varying growth paradigms. One approach should focus on identifying and characterizing myofibrillar ATPase activity and thermostability across species. In addition, an in-depth analysis of myosin hc genes in several teleost species, paying particular interest to possible "growth isoform genes," is warranted. A better understanding of how fish muscle adapts to varying environmental factors will inevitably lead to a better understanding of growth in general.

Importance of Muscle Growth to Aquaculture

Understanding the regulation of muscle growth in fish is important for two main reasons. The first is obvious in that many fish species are commercially relevant. Improvement of the aquaculture industry is vital for the advancement of the industry and an increase in high-quality meat products for consumers. Second, many commercially relevant species exhibit indeterminate growth and subsequent hyperplastic growth. This is important for biomedical relevance, as terrestrial vertebrates do not exhibit hyperplastic growth as adults and only recruit satellite cells following injury. In severe cases of sarcopenia, this system is even deficient and wasting occurs. By improving our

understanding of what mechanisms regulate cell fate for hyperplastic growth, we will be able to improve and invent strategies for enhanced fish growth and production, as well as strategies for therapeutic purposes for human muscle diseases.

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Chapter 12
Microbial Genomics of Aquaculture Pathogens

Gregory D. Wiens

Background

Infectious disease causes substantial economic loss in aquaculture and is a factor limiting production of some species. Given increasing worldwide demand for seafood, there is an escalating need for research to identify solutions to fish health problems (Bondad-Reantaso et al. 2005). It is widely recognized that disease is an outcome of complex interactions between pathogen abundance/virulence, host susceptibility, and changing or unfavorable environmental conditions. For these reasons, a comprehensive understanding of disease relies on the ability of multidisciplinary research teams to understand pathogen and host biology and to integrate these findings within the context of complex and changing environmental variables. With the advent of pathogen and host genome sequencing projects, a flood of new information is now available facilitating rapid progress in fish health research. With this information, putative virulence factors and vaccine targets are now much easier to identify and study at the transcriptional and proteomic levels.

Herein, the technical and theoretical aspects of microbial genomics are reviewed, concentrating on bacterial genomics, and how this information can be integrated to help identify virulence factors and increase vaccine development to improve fish health. This improved understanding is dependent on progress in rapid sequencing of pathogen genomes, improved microbial and host pathway analyses, and linkage via bioinformatics of host genotype and phenotype data sets. While full integration at the molecular level is still a distant goal, this chapter covers the molecular techniques associated with these advances, how to access newly developed databases, and finally how the synthesis of this information may be combined into practical approaches in the future.

Examples from rainbow trout aquaculture and associated bacterial pathogens will be used to illustrate the basic principles of this approach. Due to the long history of trout culture worldwide, a large amount of data has been collected on production parameters since the 1970s. The current figures for rainbow trout culture in the USA will be used as an example of the economic impact of disease on aquaculture. Over the past 10 years, loss in the US rainbow trout industry has been surprisingly consistent, and the annual number of fish lost due to all causes has averaged about 32 million fish per year (NASS 2007). Disease accounted for 77% of this loss (ranging between 51 and 90%) (Figure 12.1b). Disease-related loss can be direct (due to mortality), but it can also be indirect via reduced growth and feed conversion, impaired product quality, and shipping restrictions for fish and fish products. The yearly economic costs of direct and indirect losses due to disease are difficult to calculate precisely. Annual

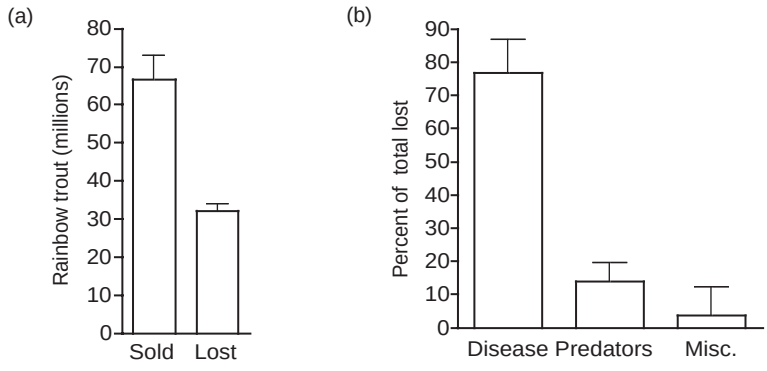

Figure 12.1. Impact of infectious disease on US rainbow trout production. Data shown were obtained from the National Agricultural Statistics Service reporting years 1996 through 2007. Yearly loss data can be obtained from http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1172. (a) Trout sold are reported in millions and include sizes ranging from 1″ through over 12″. (b) Percentage of total trout lost listed by category.

sales of rainbow trout in the USA by private industry are about $75–95 million per year (NASS 2007), with fish stocking contributing an additional estimated $325 million per year in total economic output (Caudill 2005). Thus, based on the estimate of 30% of rainbow trout production lost to disease, it is clear that a reduction in infectious disease burden would have a significant economic impact for US aquaculture and most likely worldwide.

We believe there is considerable room for improvement in trout aquaculture even though it is a relatively mature industry, as estimated loss is much higher than that for other domesticated animal production systems including poultry, pork, and beef cattle. Improved disease control is likely even more important in rapidly growing aquaculture sectors such as Atlantic salmon farming. Epizootics of infectious salmon anemia have severely impacted US, Norwegian, and Chilean production capacity. Recently, infectious disease-related losses for Chilean salmon aquaculture are estimated to amount to nearly 1 billion dollars by 2011. If disease prevalence can be reduced in the Northern Hemisphere, it may also significantly benefit wild fish populations. Recently, there has been considerable controversy over the magnitude of sea-louse infestations surrounding net-pen cages and their amplification, transmission, and associated mortality for wild salmonid populations migrating near net-pen cultures (Hansen et al. 2007; Krkosek et al. 2007). Another pathogen that has limited culture of Pacific salmon is *Renibacterium salmoninarum* (the causative agent of bacterial kidney disease). Losses of Coho and Chinook salmon from this disease were one reason why Atlantic salmon was chosen for net-pen culture in the Pacific Northwest instead of native salmonids. It is likely that pathogen genomics will play an important role in understanding each of these host–pathogen relationships. It should be noted that each host–pathogen system likely has unique features, and that in most cases, multipronged strategies are required to reduce disease. Aquaculture faces unique challenges such as intensive water reuse not common to other animal production systems and water movement (i.e., river and tidal flow) that may serve as unique challenges in preventing the transmission of disease-causing agents.

Current Status of What Can Be Done

Pathogen Genomics

Molecular advances in genomics have greatly expanded our understanding of pathogen biology. Whole genome sequencing is now a key first step in investigations of the molecular basis of pathogenesis. Pathogen "genomics" first started with the sequencing of viral genomes over 30 years ago. The first sequence of a viral DNA genome was reported in 1977 (Sanger et al. 1977), while the completed sequence of the first bacterial genome, *Haemophilus influenzae*, was reported 18 years later (Fleischmann et al. 1995). Since the completion of the first prokaryotic genome sequence, the number and rate of microbial genomes being completed have grown rapidly (Figure 12.2). As of 2009, there are a total of 977 completed genome projects comprising 817 bacterial, 56 archaeal, and 104 eukaryal genomes according to the Genomes OnLine database v 2.0 (http://www.genomesonline.org/). Twelve completed bacterial genomes and fourteen ongoing projects involve pathogens related to disease in fish (Table 12.1). The increased rate of genome completion has corresponded to a dramatic fall in sequencing costs to less than 2 cents per finished (and assembled) base pair. This reduction in cost has come about due to advances in methodologies and to economies of scale that have occurred at the large genome sequencing centers. How bacterial genomes are being sequenced, genes identified, annotated, and compared are reviewed in this chapter.

Whole-Genome Shotgun Method

The predominant method for determining a genome sequence is by the whole-genome shotgun method (Smith 2004). This method is based on the sequencing of randomly chosen fragments of DNA from the target organism (Figure 12.3). Bacterial genomes

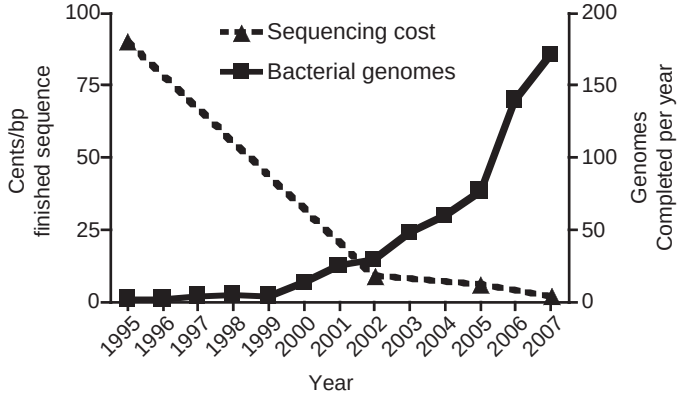

Figure 12.2. Increase in the number of publicly available genome sequences in relationship to decreasing sequencing costs. The numbers of completed genome projects are from the Genomes online database (http://www.genomesonline.org/).

Table 12.1. Completed and ongoing genome sequencing projects of fish pathogens.

	Disease	Consortium	RefSeq	Genome size (nt)
Aeromonas hydrophila ATCC7966	Motile aeromonas	TIGR/University of Maryland	NC_008570	4,744,448
Aeromonas salmonicida salmonicida A449	Furunculosis	NRC-IMB	NC_009348	4,702,402
Edwardsiella ictaluri 93–146	Enteric septicemia of catfish	University of Oklahoma/ Mississippi State University	N.A.[1]	N.A.
Edwardsiella tarda EIB202	Septicemia/wound infection	Chinese National Human Genome Center	N.A.	N.A.
Edwardsiella tarda ATCC23685	Septicemia/wound infection	Washington University	N.A.	N.A.
Flavobacterium branchiophilum FL15	Gill disease	INRA, Genoscope	N.A.	N.A.
Flavobacterium columnare ATCC 49512	Columnaris	University of Oklahoma/Mississippi State University	N.A.	N.A.
Flavobacterium johnsoniae UW101	Skin lesions	Joint Genome Institute	NC_009441	6,096,872
Flavobacterium psychrophilum CSF 259-93	Cold-water disease	IG/NCCCWA	N.A.	N.A.
Flavobacterium psychrophilum JIP02/86	Cold-water disease	INRA	NC_009613	2,861,988
Lactococcus garvieae ATCC 49156 and Lg2	Lactococcosis	Kitasato University	N.A.	N.A.
Microcystis aeruginosa NIES-843	Cyanobacterial hepatotoxicosis	Kazusa DNA Research Inst.	NC_010296	5,842,795
Moraxella catarrhalis	Furunculosis	Genome Therapeutics	N.A.	N.A.
Moraxella catarrhalis	Furunculosis	NRC-IMB	N.A.	N.A.
Mycobacterium marinum M	Mycobacteriosis	Sanger/UW/IP/MU/UT	NC_010612	6,636,827
Mycoplasma mobile	Unknown, isolated from gill of tench	Broad Institute	NC_006908	777,079
Renibacterium salmoninarum ATCC 33209	Bacterial kidney disease	UW/NOAA/NCCCWA/OSU	NC_010168	3,155,250

Streptococcus iniae 9117	Streptococcicosis	BMC-HGSI	N.A.	N.A.
Vibrio anguillarum	Vibriosis	OHSU	N.A.	N.A.
Vibrio salmonicida LFI1238	Cold-water vibriosis	Sanger/University of Tromso	NC_011312, NC_011313	3,325,165 1,206,461
Vibrio splendidus LGP 32	Vibriosis	Institut Pasteur	N.A.	N.A.
Vibrio splendidus 12B01	Vibriosis	MIT, J. Craig Venter Institute	NZ_AAMR00000000	5,596,386
Vibrio vulnificus CMCP6	Septicemia	Chonnam National University, South Korea	NC_004460, NC_004459	1,844,853, 3,281,944
Vibrio vulnificus YJ016	Septicemia	Yang-Ming University, Taiwan	NC_005139, NC_005140	1,857,073, 3,354,505
Yersinia ruckeri ATCC 29473	Enteric red mouth disease	Naval Medical Research Center	N.A.	N.A.
Spironucleus vortens	Hole-in-the-head disease	JGI, University of California, Berkeley	N.A.	N.A.

Project data were obtained from genomes-online database as of June 18, 2008. Genomes are listed alphabetically and further links to genome projects can be obtained by going to NCBI PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez), selecting "genome" from the pulldown menu and searching for the GenBank accession number. Preliminary sequence data from most genome projects can also be obtained directly from the investigators or project Web sites such as for the *Renibacterium* genome project (http://www.nwfsc.noaa.gov/research/divisions/reutd/fhm/rs-genome/index.html). However, for preliminary data it must be kept in mind the higher probability of sequence and assembly errors.

[1]N.A., not yet available.

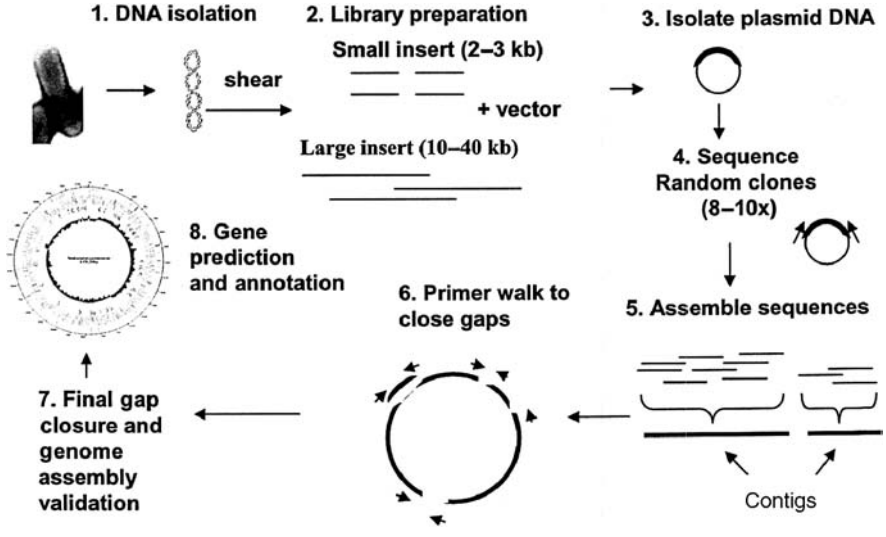

Figure 12.3. Schematic diagram of steps in whole-genome shotgun sequencing. *Step 1* involves growing the microbe from a single colony in pure culture and preparing enough DNA for library construction (>500 μg of high-purity DNA). The clone should also be verified for fish virulence prior to DNA extraction and sufficient frozen stocks of viable bacteria and purified DNA available for future reference. *Step 2* involves shearing (small insert) or partial DNA digestion (*Sau*3A, large insert) and cloning DNA into vectors (i.e., pUC19 or pGEM-3Z for small insert or pCCFOS fosmid vector for large insert). *Step 3* involves the picking of colonies that contain inserts and vector providing antibiotic resistance. This is done using automated colony picking robots. Colonies are grown overnight and then plasmid DNA isolated from each colony. *Step 4*, purified DNA is sequenced using forward and reverse primers to generate pair-end reads. Sanger sequencing using fluorescent terminator dye technology and chromatograms are edited for vector sequence and quality trimmed based on Phred quality score information. No insert vector reads and other known contaminating sequences (i.e., *Escherichia coli*) are screened from the data set. *Step 5* involves the assembly of the data into contiguous sequences known as "contigs" using programs such as Phred/Phrap/Cross_match developed at Washington University. *Step 6* involves the closing of both small gaps using the autofinish program in Consed. *Step 7* involves the final gap closure and experimental validation of the assembly. *Step 8* is the bioinformatic identification of open reading frames and the annotation of putative genes.

range in size from as small as 0.2 million base pairs (endosymbionts that have lost many metabolic pathways) to as large as 13 million base pairs. The size of the average bacterial genome is about 3.5 million base pairs. Since current Sanger sequencing technology generates only 500–1,000 bp of sequence per sequencing reaction, a large number of fragments need to be sequenced to cover a whole genome. To carry out this type of sequencing, DNA from the organism of interest is first purified and mechanically sheared or enzymatically digested. DNA pieces of defined length are then isolated and cloned. Two different size libraries are usually prepared: a small-insert library (with inserts of about 2–3 kb) and a large-insert library (10–40 kb, the size depending on the vector used). Single bacterial colonies are then picked, plasmid DNA extracted, and both ends of the inserted DNA sequenced to produce

"paired-end reads." Sequencing, until recently, had been done by conventional Sanger dye-terminator methodology, but a number of newer technologies are coming online and are covered later in the chapter. Enough clones are sequenced so that nearly all base pairs in the genome are determined multiple times. Using Sanger sequencing technology, the amount of sequence needed at this stage is about 7–10 × the size of the genome. For example, if a genome is 3 million base pairs in length, then 21–30 million base pairs of sequence need to be determined. Additional sequencing of clones, at this point, adds very little new sequence in relationship to cost. Less than 1 × genome coverage from the large-insert library is determined to facilitate the genome assembly (see below). A standard Sanger sequencing quality metric is termed Q20. Q20 is provided by the sequence trace analysis program Phred that indicates sequence that has an average of 1 error in 100 bp (Ewing and Green 1998; Ewing et al. 1998). Since there are on average seven- to ten-fold sequence coverage over each base pair, each with an error of 1 in 100, then the final error rate at the consensus position is usually less than 1 per 10,000 bp (99.99% accuracy) and probably closer to 1 per 100,000 bp (Fraser et al. 2002).

Genome Assembly and Gap Closure

Once the random sequencing phase is complete, all the sequences are assembled together using programs such as Phred/Phrap/Cross_match and are visualized in Consed (Gordon et al. 1998). Basically, these programs align the individual sequences into contiguous sequences called "contigs." Matching sequence typically needs to have about 30 bp or more overlap to be assembled, although assembly parameters can be changed to optimize sequence assembly and to take into account sequence quality. It should also be noted that a number of additional assembly programs are available and will likely continue to be modified as sequencing methods change and improve (Pop et al. 2004; Sommer et al. 2007; Pop and Salzberg 2008). After the shotgun sequencing phase, the assembly usually consists of about 100 contigs per megabase (Mb) of genome sequence. So, for a 3.5-Mb genome there will be about 350 fragments that need to be ordered into the chromosome(s). The sequence quality and contiguity is improved by carrying out multiple rounds of experiments designed by the Autofinish tool in Consed (Gordon et al. 2001). The Autofinish design experiments primarily utilize small-insert clones to improve the sequence quality and/or close gaps. Following three to six rounds of Autofinish, manual finishing utilizing small as well as large insert clones is performed. This involves (1) use of specialized sequencing chemistries to sequence difficult regions, (2) polymerase chain reaction (PCR) amplification and sequencing of specific targeted regions, (3) transposon mutagenesis of the small-insert clones followed by sequencing to fix misassembled or difficult to assemble regions, and (4) shotgun sequencing of the targeted fosmid (similar to a cosmid but are based on the bacterial F-plasmid) clones to fix long-range misassemblies in the assembled genome. Selected clones from the small- and large-insert libraries are informative if they "bridge" between contigs. Since the approximate size of each clone is known, the approximate size of the gap can be estimated. For the *R. salmoninarum* genome, after the initial shotgun sequencing stage, sequence data were assembled into approximately 195 contigs. Primer walking required 8,968 reads and the complete sequencing of 29 fosmid clones (22,272 reads) to close all gaps.

The final assembly consisted of 51,799 sequence reads with a final genome size of 3,155,250 bp (Wiens et al. 2008). The gap closure stage is the most time-consuming and expensive stage, as human intervention is required and costs can equal or exceed the primary shotgun sequencing phase. Given that the final assembly is based on a computer prediction, experimental verification of the final assembly is required. For the *R. salmoninarum* genome project, the assembly was validated by two independent methods. The gross scale was verified by agreement between the virtual fingerprint pattern of the genome from *Pme*I, *Pac*I, or *Swa*I restriction enzyme digestions, and the experimentally determined restriction fragment sizes determined by pulsed-field gel electrophoresis. For a finer scale (1 kb), fingerprint data were generated from the paired-end-sequences of 768 fosmid clones by digesting with three independent restriction enzymes, *Bgl*II, *Hind*III, and *Pst*I, that generated 2,256 of the theoretical 2,304 fragments (97.9%) predicted from these clones. The virtual and experimental fingerprint patterns (having ends anchored in the genome sequence) were compared using the SeqTile software tools developed at the University of Washington Genome Center (Wood et al. 2001; Hendrickson et al. 2004). After some corrections, complete correspondence between the virtual and experimentally derived fingerprint pattern of the genome was obtained, thereby validating the final genome assembly. Other methods for validating genome sequences include long-range PCR (Duchaud et al. 2007) and optical restriction mapping (Latreille et al. 2007; Nagarajan et al. 2008). Another alternative is not to close the genome or validate the assembly and then submit these data to GenBank as a "draft genome." Draft genome sequence contains a higher number of sequence errors and misassemblies. However, a great deal can be learned from draft-quality genomes without the associated expense of determining every base pair in the genome. For a good discussion of arguments for closing, see Fraser et al. (2002) and for the advantages of draft sequencing, see Branscomb and Predki (2002).

Automated and Manual Annotation

Once assembled sequence is available, the next step is to identify genes and begin annotation. The overall goal of annotation is to describe gene function as accurately as possible as a means to derive biological meaning from sequence data (White 2004). Annotation includes assigning gene product names, functional characteristics of gene products, physical characteristics of gene/protein products, and can include an overall metabolic profile of the organism. Annotation accuracy and consistency between genome projects is highly variable as standards and criteria vary between locations and also depend on the skill of the principal investigators (or often students), the available resources and time, and ability to delve into related literature. Annotation is much easier if a similar microorganism has been sequenced for which high-quality annotation is available. It should be kept in mind that annotation of most genes is only a hypothesis and that very few genes in most sequenced genomes have actual experimental evidence supporting their existence. Also, as more genomes are sequenced and more gene function data accumulate, reannotation of the same genome sequence can greatly improve the quality and accuracy. In fact, it has been proposed that it is easier to annotate 1,000 genomes than 1 (Overbeek et al. 2005). This is because common genes are easy to identify, compare, and annotate across many genomes,

and gene synteny (gene order between genomes) is a powerful tool for identifying gene orthologs (same gene in different species). Furthermore, the novel genes become readily apparent when there is nothing similar in the database. The process of manually inspecting each open reading frame (ORF), while time consuming, provides the investigator a high degree of familiarity with a genome and is the source of many new ideas and hypotheses for future wet-laboratory investigations.

Current high-throughput genome annotation uses a combination of comparative (sequence similarity comparisons) and noncomparative (ab initio gene prediction algorithms) methods to identify protein-coding genes in genome sequences. During the annotation process, the origin of chromosome replication is identified based on the context of genes frequently observed near the putative origin and the skewed orientation of oligomers around the origin (Salzberg et al. 1998b). For the majority of bacterial species, *dnaA*, a gene whose product binds to the origin of replication, is located close to the origin. A change in the skew of GC bases is also used to help identify the origin, and it is calculated as the difference between the number of Gs and Cs normalized to the G+C content (see Guy and Roten 2004; Guy et al. 2005 for additional discussion of origin identification). A Web-based program, Ori-Finder (http://tubic.tju.edu.cn/Ori-Finder/), is available to search unannotated genomes (Gao and Zhang 2008), and results can be compared to DoriC, a database of all known oriC regions in bacterial genomes (Gao and Zhang 2007). A base pair at the origin is labeled as nucleotide 1 and the downstream base pairs are numbered clockwise around the genome (most bacteria have circular chromosomes). Some of the general features annotated in genomes include ribosomal RNA genes (tRNAscan; http://selab.janelia.org/tRNAscan-SE/ and RNAmmer; http://www.cbs.dtu.dk/services/RNAmmer/), insertion elements (ISFinder database; http://www-is.biotoul.fr/), repeated regions (Genome Atlas Database; http://www.cbs.dtu.dk/services/GenomeAtlas/), and integrated phage genomes (PhageFinder: http://phage-finder.sourceforge.net/).

ORFs are identified along the sequence by a number of computer programs. An ORF is a stretch of DNA sequence that begins following a stop codon (TAA, TAG, or TGA) and then ends at the next stop codon and meets a minimum size (>90 bp depending on the program). It can be in any of the six possible reading frames of DNA. ORFs can occur by chance and a "gene" is the coding sequence within an ORF that has a defined "start" codon (ATG, GTG, or TTG) and a stop codon. Glimmer (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi) is a commonly used program that assigns probabilities to potential coding regions using interpolated Markov models based on a training set of known genes (Salzberg et al. 1998a; Delcher et al. 1999). Microbial genes rarely overlap so this is a criterion for excluding many ORFs (Salzberg and Delcher 2004). Predicted coding regions are then searched against databases of known proteins and assigned automated annotations. There are a variety of graphical interfaces for viewing and annotating genes that include commercial systems such as ERGO (Overbeek et al. 2003) and Pendant-Pro, and open source systems such as Artemis (Berriman and Rutherford 2003), GenDB and REGANOR (Meyer et al. 2003; Linke et al. 2006), Manatee (White 2004), AGMIAL (Bryson et al. 2006), and RAST (Aziz et al. 2008). Describing these systems is beyond the scope of this chapter; however, an example of one graphical interface is provided. The protein page of a *R. salmoninarum* gene, *msa1*, is shown in Figure 12.4. Each page contains relevant gene/protein statistics, similarity to other genes, genome location, and further tools for bioinformatic investigation.

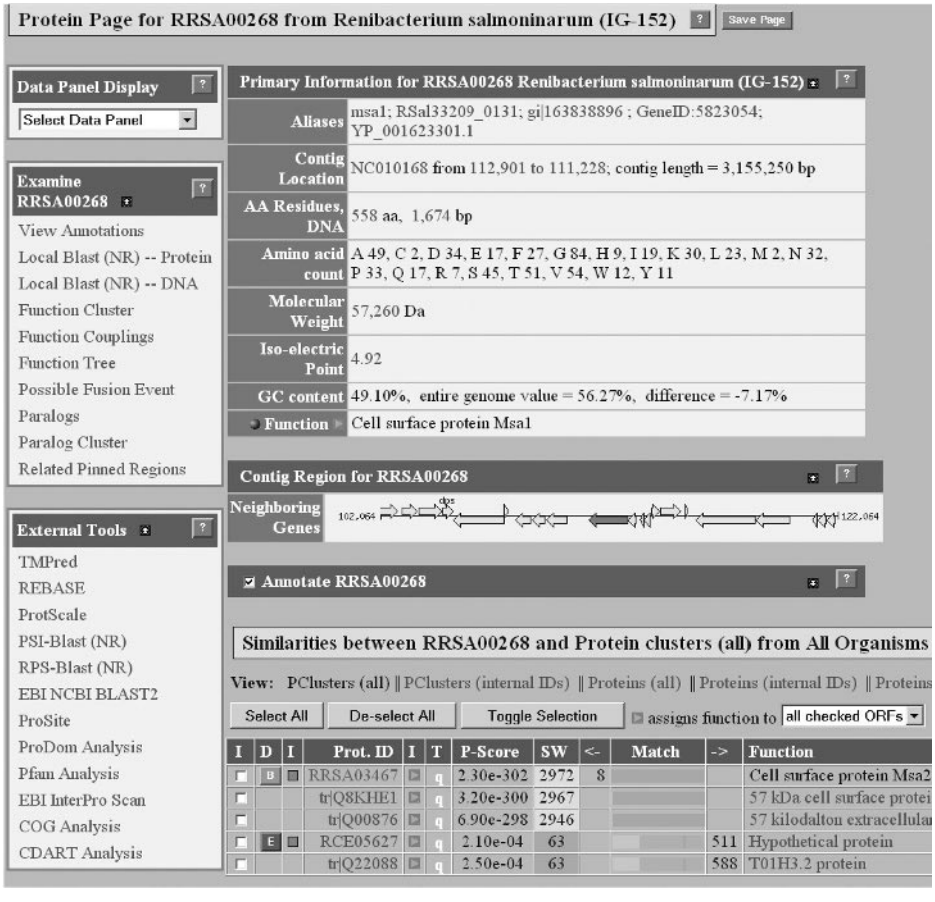

Figure 12.4. ERGO graphical interface of the *msa1* gene from the *R. salmoninarum* genome. Each data page contains information on the predicted protein, the location in the genome, similarities to other proteins, and bioinformatic links for further analysis.

Bioinformatic Tools for Annotation

Once ORFs are identified, a variety of bioinformatic tools can be utilized to determine their relationship to other known genes, and thereby leading to a proposed annotation. It should be noted that automated annotation is usually performed multiple times. For example, if performed after the shotgun assembly stage for an initial inspection of the data, the automated annotation can help close some gaps, and thus the final closed genome sequence is obtained. For the *R. salmoninarum* genome project, the annotation workflow used is described in Figure 12.5. Each ORF was searched against all other known proteins using BLASTP or PSI-BLAST searches of the nonredundant protein database at NCBI. If a hit having a high score was obtained, and there were multiple lines of evidence (i.e., PFAM, TIGRFAM, and CDD hits) indicating a conserved domain and/or specific function, then the predicted protein was given a specific name, gene symbol, and finally an Enzyme Commission (EC) number if it had an enzymatic function. EC numbering is a numerical classification scheme for

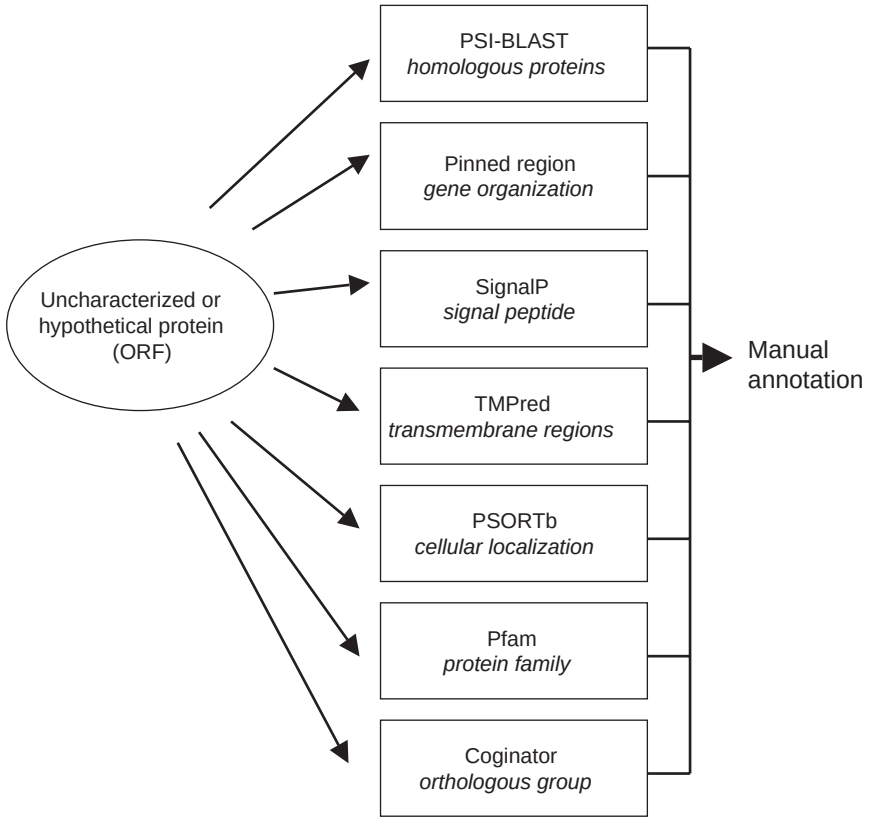

Figure 12.5. Annotation workflow.

enzymes based on the chemical reactions catalyzed (i.e., Glucokinase with EC number 2.7.1.2 converts ATP and D-glucose into ADP and D-glucose 6-phosphate). To transfer annotation from an experimentally characterized protein, the cutoff criteria used by TIGR (The Institute for Genomic Research) annotators are that a protein should have >35% amino acid identify over >80% length. This may seem like a pretty relaxed requirement, but for most genes in a new genome sequence, even less information is available. In such cases, the gene role/identity is annotated with a descriptive term such as "putative transporter" or "transcriptional regulator, MarR family." If no domains are identified, then the predicted cellular location is examined and it can be noted if the protein contains a transmembrane region or appears to be secreted. ORFs similar to other hypothetical genes were annotated as "conserved hypothetical protein," or if they contained no similarities they were annotated as "hypothetical protein." Typically, the "hypothetical" ORFs were short ORFs encoding putative proteins of <50 amino acids. Hypothetical ORFs are potential genes with the least supporting evidence and should be considered suspect until wet-laboratory experiments confirm gene transcription and/or function. There was an additional challenge to annotate the *R. salmoninarum* genome as there were a high number of partial gene sequences, each similar to different parts of a full-length protein from other genomes. These were often adjacent to one another, and separated by a stop codon or frameshift.

Examination of the primary sequence trace data demonstrated that these were not sequencing artifacts, and these same changes were amplified from bacteria isolated from fish lesions. Thus, it was concluded that these partial genes were not artifacts due to laboratory propagation of the bacterium but rather were putative pseudogenes. The subsequent availability of two *Arthrobacter* genomes (Mongodin et al. 2006) to look for synteny using the "pinned region function" in ERGO, greatly facilitated the identification of these partial genes.

For additional discussion of the annotation process, the reader is referred to an excellent review of TIGR standard operating procedures used to assign gene function (White 2004). Annotation training classes are also available at http://www.jcvi.org/cms/education/prodev/prok/ and http://ae.igs.umaryland.edu/cgi/index.cgi. Additional specific annotation tools for subsets of genes include ABC transporters (ABCISSE database; http://www.pasteur.fr/recherche/unites/pmtg/abc/) and proteases (MEROPS database; http://merops.sanger.ac.uk/).

Problems/Caveats of Genomic Data

First, it must be kept in mind that sequencing errors and assembly errors are present in genome sequence data (Salzberg and Yorke 2005). Most assembly errors arise from repeated-sequence regions within a genome (Phillippy et al. 2008). Repeated sequences include ribosomal RNA genes, insertion elements (jumping genes), and other unique types of repetitive elements. Often these repeats are collapsed, and thus more genes exist in the genome than in the final assembly. Conversely, the presence of numerous expansion artifacts was recently identified in comparative analysis and reannotation of the *Vibrio fischeri* genome (Mandel et al. 2008). These expansions were the product of misincorporation of extraneous sequence into the published assembly, leading to the appearance of duplicated DNA that occurred only in a single copy. A total of 14 extra regions of DNA were identified consisting of 0.2% of the 4.3 Mb genome. It is likely that the automated gene calling software makes mistakes, and by one estimate, many genomes are "overannotated" by as much as 20% (Ussery and Hallin 2004). In addition, it has been noticed that using different ORF calling software can make a considerable difference in the number of genes identified. This is more of a problem with high G + C content microbes as translational start site accuracy is estimated at only 75% (White 2004). In the *R. salmoninarum* genome project, Integrated Genomics automated ORF calling was compared to TIGR automated ORF calling and found that 90% ($n = 3{,}340$) of the predicted genes contained the same stop site; however, there were considerable differences in start site prediction and the total numbers of ORFs identified. ERGO predicted 180 unique ORFs that TIGR did not predict, and TIGR predicted 210 unique ORFs that ERGO did not. It is unclear whether any of these sequences are actual ORFs, and most lacked matches to ORFs in any other microorganism, and thus were considered "hypothetical." Of the 3,340 ORFs predicted to have the same stop codon, 31% of these were predicted to have different start sites. This is significant as it can impact signal peptide prediction programs that predict cleavage characteristics within a defined region of the amino terminus. These varying start-site predictions underscore the need for experimental validation of ORFs using high-throughput methods.

In addition, some types of genes, such as antisense transcripts, are not well predicted by standard programs (Silby and Levy 2008) and, as such, are absent from GenBank submissions. The recent identification by Illumina sequencing of 5′ and 3′ transcriptional gene boundaries of yeast genes greatly improved gene prediction models and also identified new genes (Nagalakshmi et al. 2008). A similar transcriptional mapping approach is likely to be highly informative for bacterial species as well.

Another common mistake is transitive annotation error. This occurs when experimentally characterized gene A is similar to gene B, and then annotation is transferred to B. Subsequently, gene B is found to be similar to C, but C has little relationship to the original gene A for which the experimental data were obtained (Salzberg 2007). This can be prevented only by using a well-curated data set for annotation. However, no comprehensive and standardized data set now exists, thus investigators are left on their own to evaluate experimental evidence behind each annotation. For some model organisms like *Escherichia coli*, extensive functional annotation and updated web-based databases exist such as EcoCyc (http://ecocyc.org/). Solutions to these problems also include having one annotator for one "subsystem" (such as transcription factors or transporters) to increase annotation standardization and accuracy (Aziz et al. 2008). A proposed solution for out-of-date annotation and GenBank stasis is to utilize a model similar to the online encyclopedia, Wikipedia, in which research communities constantly reannotate genomes (Salzberg 2007). Extensive annotation and sequence information of select human pathogen groups is also available online at the National Microbial Pathogen Data Resource (http://www.nmpdr.org/cur/FIG/wiki/view.cgi/Main/WebHome) (McNeil et al. 2007). While it is unclear how the annotation process will evolve in the future, it is considered a worthwhile exercise for any new researcher or graduate student, who plans to work on a sequenced fish pathogen, to first go through the process of gene prediction and reannotation of the genome sequence of interest. This will help clarify where limitations or discrepancies exist and is also likely to uncover many aspects that the original investigator did not have the time or tools to identify.

Post-Sanger Strategies for De Novo Sequencing

Recent advances in sequencing chemistry, and novel sequencing strategies, promise to radically change how genomics is performed in the near future (Medini et al. 2008). The most widely used technology is that of 454 sequencing methodology (Margulies et al. 2005). According to the genomes-online database, 454 technologies are being used either alone or in combination with Sanger sequencing in over 190 genome projects. In this technology, DNA is sheared, and oligonucleotide adaptors are attached to the DNA fragments. One adaptor-linked DNA fragment is then attached to one bead and multiple copies of the single DNA are synthesized within a droplet of an oil–water emulsion by PCR. Each product is then attached to the bead and each bead is inserted into a picoliter-sized well in a fabricated substrate. The sequence of the DNA attached to the bead is determined by parallel pyrosequencing. In this chemistry, the measurement of each nucleotide incorporated is the release of inorganic pyrophosphate. Inorganic phosphate is then converted into ATP and finally into light by the enzyme luciferase. A photodetector measures the light emitted from each well and these data are converted into sequence reads. Currently, sequence reads average

250 bp in length with the possibility of obtaining paired-end reads using the GS-FLX system. In the near future, read lengths of >400 bp are proposed. The drawbacks of this technology are the collapsing of homopolymeric repeats. However, a significant advantage is that there is no cloning step, thus solving some vexing, uncloneable gaps due to the fragments being toxic for *E. coli* growth. Two other rapid sequencing technologies are the SOLEXA/Illumina and SOLiD technologies, both of which currently produce only short reads of 25–40 bp (Medini et al. 2008). The shortness of these reads makes it difficult to assemble the data into a completed genome and currently this technology is best suited for resequencing and single nucleotide polymorphism discovery.

There is little doubt that some of these newer sequencing technologies used either alone or in combination with standard Sanger technology will radically reduce the cost of determining a bacterial genome sequence. It is envisioned that in the next 10 years, genome sequencing cost may be relatively trivial compared to today and that it will be routinely performed on new isolates of aquaculture disease agents. It is predicted that the major difficulty will be the downstream bioinformatic process of sequence that includes the information storage (terabytes of data), data retrieval, and the development of integrated tools meshing data from disparate databases. New methods are needed to produce automated and up-to-date annotation of sequence and to distill the relevant biological information to fish health workers. One solution is a web-based tool specific for traditional agricultural species named AgBase. This is a curated and public resource that seeks to link functional genomics to systems biology (McCarthy et al. 2006, 2007a, 2007b). There is also a considerable need for researcher training to stay up-to-date with the fast-changing fields of bioinformatics and data management.

Comparative Resequencing

Once a complete genome sequence is available, it is possible to identify differences between closely related strains using comparative genome sequencing (Albert et al. 2005). This utilizes oligonucleotide microarrays to determine regions of difference in a test genome compared to a reference genome. This technique uses a two-step process in which mutations are first mapped by microarray, and then in the second step, they are subjected to finer mapping and sequencing using microarrays. In the initial microarray, probes (29–32 mers) spanning the entire genomes are synthesized with 7 or 8 bp spacing from the start of one probe to the start of the next probe. Both the reference genome DNA and DNA from the test genome are hybridized to the microarrays, and the ratio of signal intensity between the reference and test genome determined. Regions of difference are then subjected to finer-scale mapping. A second microarray is then specifically created to interrogate only the regions of interest at the level of single base-pair resolution. Probes with all four possible nucleotides are synthesized for each position of interest for both strands (8 probes per position). For single-base polymorphisms, the pattern of hybridization intensities is different for the match versus the mismatch. This technique does not conclusively resolve sequence differences such as insertions/deletions or differences of more than 1 bp, but these regions can generally be amplified by PCR and determined by conventional sequencing. This technology works best if there are only minor differences (<0.01%)

between the test and reference genome, and it has been successfully applied when the sequenced strain was selected for a new phenotype such as drug resistance or lack of virulence (Albert et al. 2005). There are several drawbacks to this technology: (1) large rearrangements are not detected, (2) repeated regions are problematic, thereby limiting their utility for large families of paralogues, and (3) mutations in regions of high secondary structure may not be detected (Herring and Palsson 2007). Costs for the first stage of microarray mapping are ~$1,000 per Mb with resequencing costs dependent on the number of regions of interest.

Comparative Genomics

Once a genome is determined, it is of high interest to make global comparisons to other sequenced genomes and this endeavor is termed comparative genomics. The large number of publically available microbial genomes allows for comparisons across all taxonomic levels, from kingdom to strain comparisons. There are several public and one commercial broad-species comparative genomic platforms. These are the Comprehensive Microbial Resource (Peterson et al. 2001) (http://www.tigr.org); the Department of Energy, Joint Genome Institute Comparative Microbial Resource (http://img.jgi.doe.gov) (Markowitz et al. 2006, 2008); and Integrated Genomics database (ERGO web site) (Overbeek et al. 2003). For detailed description of each system and capabilities, the reader is referred to the web site and associated publication(s). The three resources have the following in common: broad-based data retrievals on protein properties, functional role assignment, precalculated orthologs identified from bidirectional best hits, paralogue prediction, and visualization of whole-genome synteny. Each system not only uses internal automated annotation schema to assign ORFs and functional assignments but also maintains links to original data sets. Functional annotations and metabolic pathway information are added to each genome when entered into these databases. Users can add their own ORF and pathway annotations to both IMG and ERGO systems.

Future Directions

How can these techniques become integrated with existing fish health research and what kind of progress can be expected? Two areas relevant to fish health are discussed: (1) understanding pathogenesis and (2) facilitating vaccine development.

Application to Studying Pathogenesis

It is evident that having an annotated catalogue of genes quickly provides information about similarities and differences to other known pathogens. For example, having the sequence of *R. salmoninarum* allowed us to examine how closely it was related to pathogenic *Mycobacteria* spp. Both belong to the order Actinomycetales (high G + C, Gram-positive bacteria), are slow growing intracellular pathogens, and induce granulomatous lesions in host tissues. However, it was quickly apparent from comparative genomic analyses that most of the known mycobacterial virulence genes were absent

from the *R. salmoninarum* genome. Interestingly, *R. salmoninarum* was most similar to the soil microorganisms in the genus *Arthrobacter*, that are not known pathogens, and we identified a large set of common core genes and a smaller set of unique genes (Wiens et al. 2008). The unique *R. salmoninarum* genes are of high interest for further functional analysis and their potential role in pathogenesis.

Virulence genes in some microorganisms are clustered together in the genome. These clusters are referred to as "pathogenicity islands." They often have sequence characteristics that suggest that they were transferred from another microorganism by a process known as horizontal gene flow, which can occur via phage, plasmid, or natural DNA uptake. Features commonly associated with these islands include transposases and integrases (jumping genes), tRNAs (often insertion sites for these elements), flanking repeats, and these regions often have either a higher or a lower guanine and cytosine (G + C) percentage as compared to the genome average. There is wide variation in G + C content between microorganisms, and thus the horizontally acquired genes are often different from the host genome average. The features characteristic of pathogenicity islands can be viewed graphically, in a whole genome context, using a web-accessible service IslandPath (http://www.pathogenomics.sfu.ca/islandpath/). This web site contains all genomes submitted to GenBank, including the fish pathogen genomes listed in Table 12.1. This is a quick tool to browse a genome of interest for regions that may be candidates for wet-laboratory experiments.

Having a genome sequence allows the construction of whole-genome microarrays, and it is now common to use this as a technique to compare pathogen gene expression during growth on media or in host tissues to thereby identifying genes induced under infection-specific conditions. Additionally, mapping mutations responsible for phenotypic changes such as virulence is now feasible using comparative microarray sequencing (discussed above). This may be a useful approach for bacterial fish pathogens for which a genome is known but there are limited or difficult genetic systems (such as *R. salmoninarum* and *Flavobacterium psychrophilum*).

Integrating Microbial Genomics with Vaccine Development

An initial use of pathogen genome sequence information was the prediction of all putative cell-surface and secreted proteins. This was performed *in silico* by predicting features typical of surface-associated proteins. These include presence of features such as presence of leader peptides, outer membrane anchoring motifs and secretory leader sequences, transmembrane domains, lipoprotein signatures, and homologies to known surface proteins (Kaushik and Sehgal 2008). These targets can then be tested either alone or in combination with each other in animal models for the induction of protective immunity. This genome-based approach is referred to as reverse vaccinology and, when combined with proteomics, is a powerful approach to identifying vaccine candidates against extracellular pathogens (Movahedi and Hampson 2008). This process was first used to identify vaccine candidates from *Neisseria meningitides* serogroup B (Pizza et al. 2000) and *Streptococcus pneumoniae* (Wizemann et al. 2001). This process may be useful as well for extracellular bacterial fish pathogens for which a whole vaccine is not available or technically feasible. However, it has, to date, proven less successful with intracellular pathogens.

Summary

In summary, much basic and practical information has been, and will continue to be, learned from the complete and draft genome sequencing of fish pathogens. Given the large number of pathogens, this field is still in the early stages of development. To date, most projects have focused on viral and bacterial pathogens, and in the future, pathogens with more complex genomes are likely to be targeted. At present, there is only one project involving a eukaryal fish pathogen, *Spironucleus vortens* that causes hole-in-the-head disease. It is also likely that aquatic fungal pathogens from the genus *Saprolegnia* will be initiated given their economic importance (Phillips et al. 2008). Sea lice may be another eukaryal pathogen genome of high priority for the fish disease research community. The availability of these sequence data and that from a number of aquaculture host species promises to keep fish health researchers busy for some time.

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Chapter 13
Control of Reproduction

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Introduction

Control of reproduction is important for seed stock production, selective breeding, growth rate, feed efficiency, meat quality, and biosecurity. These needs to control reproduction differ among cultivars and even within segments of the same industry. For example, tilapia can reproduce at a very small size causing stunting from overcrowding, whereas female striped bass (*Morone saxatilis*) do not mature until they are over 3 years of age which hampers seed stock production and selective breeding. Growers of tilapia are therefore interested in means to delay or prevent sexual maturation, whereas the hybrid striped bass industry is interested in means to get female striped bass to mature early. Similarly, in salmonids, earlier maturation may be desirable for seed stock production and selective breeding efforts, whereas delayed maturation, sterility, or monosex production may be desirable for improved growth rate, feed efficiency, meat quality, and biosecurity. It is also evident from these examples that diverse aquaculture needs can often be addressed by greater control over a shared underlying reproductive process, in these particular examples, the onset of puberty. No matter the impetus for aquaculturists to want to alter reproductive events, progress in gaining control over reproductive processes, is dependent on our gaining a greater understanding of mechanisms controlling these processes. Molecular techniques have been instrumental in many recent advances in our understanding of reproductive control mechanisms in fish.

The nature of reproductive assistance sought by aquaculturists is wide in scope due to both the large number of species being cultivated and the considerable diversity in reproductive physiology among fish species. These differences in physiology hamper the adaptation of advances made in one species to another species. With more than 28,000 species of fish to consider as cultivars, it is obvious that we cannot start from scratch with each new species of interest. Instead, we must recognize the bases of shared and disparate control mechanisms among species. An example of a shared mechanism is estrogen induction of the yolk precursor, vitellogenin (Vtg) in all fishes, whereas the difference in the processing of the Vtgs in fish with pelagic versus benthic eggs is a level of disparate control mechanisms. A greater understanding of basic reproductive processes among species with varying modes of reproduction is required to recognize these underlying commonalities and differences. Fortunately, it is more than just the impetus to advance aquaculture that is driving research on fish reproduction. Attributes of fish reproductive physiology, such as large follicle size, high fecundity, and rapid reproductive rates, have made fish indispensable models for biomedical-related research that can nevertheless be exploited for aquacultural purposes.

Reproductive research helps aquaculture in primarily one of two ways. The first is to provide new tools for control of reproduction, which has often been in the form of hormone preparations or diagnostic tests. The second is to better understand the reproductive physiology of fish to better direct us on how, when, and what tools to apply in a particular situation. For instance, pituitary and gonadotropin-releasing hormone (GnRH) preparations have become important tools for induced spawning of fish, but they can work only if the fish are at the right reproductive stage to respond properly. So, a better understanding of the mechanisms of gonadotropin (GtH) and GnRH actions will enable the design of improved diagnostic tests to evaluate the fish for competence to respond to hormonal treatment.

A major advantage of molecular techniques is the relative ease in which the functioning of many reproductive processes can be addressed, at least in part, in a new species of interest. As mentioned, in addition to the shear multitude of fish species, there is greater phylogenetic diversity and variation in modes of reproduction among fishes than in the remainder of all vertebrates. Many of these species have potential for aquaculture, particularly when the ornamental fish industry is taken into account. Before molecular techniques were available, it was very difficult to address reproductive control pathways in a new potential cultivar. Histological techniques combined with measurement of conserved sex steroids such as estradiol-17β (E2) and testosterone (T) were about the only way to examine reproductive status to determine where reproduction was failing in a new species. With the advent of molecular biology, expression of just about any gene known to be a marker of reproductive stage or functional state can be measured in any species with relative ease, greatly increasing our ability to assess reproductive status in a new species. As reproductive control pathways are better characterized, critical reproductive events in fish with similar reproductive strategies can be recognized, and then it will become easier to gain control over reproduction in new species of interest.

Due to the many advances in response to the great interest in fish reproduction, it is impossible to begin to cover all molecular and cellular techniques have revealed about control mechanisms in reproduction of fishes. On the other hand, there are so many exciting findings that have yet to be exploited for aquaculture purposes that it would be a disservice to restrict this chapter only to those new procedures that have already resulted in applications. The approach chosen is to present some of the more exciting findings and imaginative exploitations of current molecular and cellular techniques as examples of the potential of this means of investigation to unlock the knowledge needed to drive future aquaculture applications. This chapter is therefore divided into various topics of aquaculture interest.

Control of Spawning and the Brain Pituitary System

Among the first steps in establishing an aquaculture industry is obtaining seed stock. Although some industries have grown to great size based on the capture of live seed stock, such as the milkfish (*Chanos chanos*) industry in Southeast Asia, most only began to take off after methods to spawn broodstock were developed. Interestingly, among the first and the largest industries are those based on fish that spawn well in captivity with little manipulation, such as carps, salmonids, and catfish. However, even in carps, which have been cultured for thousands of years, and in salmonids, for which

there have been commercial hatcheries for well over a hundred years, culturists are still trying to obtain better control over their reproduction. The main point is that even though obtaining the ability to spawn broodstock is a watershed event in an aquaculture industry, it is just a beginning.

A primary impediment to seed stock production is that many fish species do not naturally spawn in captivity. The nature and bases for these problems are many. Most often female fish will develop fully grown oocytes but fail to progress through follicle maturation (FM) and ovulation. FM consists of two stages. The first stage includes acquisition by the follicles of the capacity to produce the maturation inducing hormone (MIH) and the acquisition of oocyte maturational competence (OMC). OMC is the ability of the oocyte to respond to the MIH by entering the second stage of FM, resumption of meiosis, and cytoplasmic competence for fertilization. In addition to problems with these processes in females, males often produce less milt in captivity.

It is generally considered that many fish that fail to reproduce in captivity fail to do so because they are not receiving the proper environmental or social cues (see Zohar and Mylonas 2001). Failure to spawn naturally can be overcome by discovering how to enhance the environment of fish to provide appropriate stimuli such as by adding a plant-based spawning substrate for goldfish (*Carassius auratus*). Alternatively, fish can be induced to spawn by administration of exogenous hormones including a GtH or GnRH preparation. Other fish arrest gonadal development much earlier but in some instances are still able to be successfully treated with exogenous hormones, as with milkfish (Lee et al. 1986) and Japanese eel (*Anguilla japonica*; Ohta et al. 1997). Not surprisingly, the initial use of exogenous hormones for extended periods of time in milkfish has been replaced with improved husbandry techniques and domestication of the broodstock (Emata and Marte 1994). In the case of milkfish, the use of hormones facilitated the development of the more natural techniques. This reduction in the need for exogenous hormones, as domestication or husbandry of a species advances, is quite common.

Even after development of exogenous hormone preparations to replace endogenous hormones that are not being released properly in captivity, there is still a need to determine when and how to deliver the hormone preparations. Among the most common ways to evaluate fish as candidates for breeding or induced spawning are by feeling the belly to assess the size of the gonads and degree of hydration of the eggs, or by biopsying the ovary and evaluating follicle diameter sometimes in conjunction with morphological indices associated with FM (Rees and Harrell 1990; Hodson 1995). Spawning techniques for striped bass are a good example of techniques currently in use to assess maturation for determination of the appropriate hormone treatment. Brood fish can be induced to spawn with human chorionic GtH (hCG) injections if the oocytes are advanced to stages where the oocytes display coalescing oil droplets in their ooplasm. Often captive striped bass do not reach these stages before follicle atresia is initiated and the eggs are lost. An option is to treat fish with oocytes over 800 μm but not initiating oil droplet coalescence, with GnRH implants to induce spawning before atresia begins. Even so, many fish of adequate oocyte diameter respond poorly to GnRH implants because there is great variation among individual fish in the follicle size at which they become competent to respond to hormone treatment. Basic research on the actions of insulin-like growth factors (IGFs) led to development of a diagnostic test in which biopsied follicles are treated with IGF-I in vitro, and if the oocyte nucleus or germinal vesicle breaks down, the donor fish are ready to receive

GnRH implants (Weber et al. 2000). This approach requires only techniques commonly used by the farmer to evaluate their broodstock, biopsying follicles and staging the degree of maturity of the follicles. Similarly, in several species it was previously determined that follicles obtained by biopsy that respond to the progestin MIH in vitro indicate that the donor fish is ready for GtH or GnRH treatment to induce final maturation (e.g., Lutes et al. 1987).

Aquaculture production has benefited greatly from the use of exogenous pituitary and hypothalamic-derived hormones to induce spawning (see Zohar and Mylonas 2001). Chorulon® (Mafr. Intervet International, B.V. Boxmeer, Holland), which is hCG, Ovaprim® and Ovaplant® (Mafr. Syndel Laboratories Ltd., Qualicum Beach, BC, Canada), which are a combination of a salmon GnRH analog and the dopamine inhibitor domperidone, and Gonazan™ [Mafr. Intervet International, B.V. Boxmeer, Holland], which is a GnRH analog, are examples of drugs that are at least conditionally approved for induction of spawning of food fishes in the USA or European Union. These reagents are used in many aquaculture industries including the hybrid striped bass, European sea bass (*Dicentrarchus labrax*), and various carp and salmonid industries. However, even the use of sophisticated microencapsulated time-released pellets to deliver these preparations still amounts to a crude overriding of inhibitions to natural reproduction that are not fully understood. A greater understanding of the functioning of the brain–pituitary system would certainly lead to greater control of spawning and puberty, and improved egg quality.

Pituitary hormones were first used for induced spawning in the 1930s. Initial use involved removing the pituitaries from one or more mature fish and injecting them into another fish to induce the recipient fish to ovulate or spermiate (Houssay 1931; von Ihering 1935, 1937). This technique was refined and expanded to include acetone-dried fish pituitaries, purified mammalian GtHs including hCG, and partially purified fish GtHs. Most of these reagents are still in use today. Unfortunately, still a method does not exist to produce large quantities of pure fish GtHs, a task being currently addressed with molecular approaches.

Aquaculture was fast to make use of GnRH after its sequence was reported in 1971 (Matsuo et al. 1971; Burgus et al. 1972), for which Andrew Schally and Roger Guillemin later received the Nobel Prize in Physiology and Medicine in 1977. As early as 1974, Hirose and Ishida (1974) induced ovulation in Japanese ayu, *Plecoglossus altivelis*, with a single injection of mammalian GnRH. This was followed later by the widespread use of pelleted GnRH analogs to induce ovulation in a variety of aquaculture species (e.g., Crim and Glebe 1984; Harvey et al. 1985). Long-term administration was even shown to assist ovarian development from early gonadal stages in milkfish (Lee et al. 1986, 1987). As mentioned, commercial GnRH-based products are available. Furthermore, delivery systems have been improved including the technology to produce microsphere encapsulated GnRH that holds great promise to increase control of GnRH delivery and for use in smaller species such as ornamentals (Mylonas et al. 1995).

Although great strides in aquaculture were made due to the introduction of these products to manipulate gonadal development, there was and still is great potential to further exploit the actions of hypothalamic and pituitary factors in order to gain even greater control over reproduction. As with all systems that will be discussed, a greater understanding of how the component factors function, and even the identification of new factors that contribute to the regulation of reproduction, will form the basis for

improvements in control of fish reproduction. Molecular techniques and approaches have and will continue to play a major role in this advancement.

One area where molecular biology has been extremely useful is the identification and characterization of the components of the reproductive system. This includes the structures of ligands, receptors, binding proteins, and signaling molecules. The reasons for interest in pituitary hormone structure are multifaceted. First are the basic structure–function relationships that might provide more information as to how the GtHs are meant to function. This knowledge should help in elucidating and then correcting for GtH systems not functioning properly in captive fish. A second reason is to predict which commercially available hormones might best influence the species one is interested in spawning by comparing the structure of products to the GtHs of that species. For example, hCG works well in many fish species but not in salmonids. A third interest is to make available new pituitary hormone-based pharmaceuticals and reagents for the control of reproduction and as diagnostic tools. This includes recombinant forms of the native GtHs for a particular species, antagonists to block reproductive processes, or analogs that are less expensive, have greater activity, longer half-lives or greater specificity for the different GtH receptors. Diagnostic tools include, as examples, reagents for GtH RIAs (radioimmunoassays) or ELISAs (enzyme-linked immunosorbent assays) for identifying reproductive stage or assessing the response of fish to a certain treatment, environmental or otherwise, that an aquaculturist is attempting to use in order to manipulate reproduction.

The characterization of GtH structures is an excellent example of the introduction of molecular approaches in fish physiology. The GtH molecule is made of an alpha and beta subunit. The alpha subunit is shared among the follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyrotropin (TSH) molecules. The beta subunits are different and impart receptor specificity. The hormones are excreted from the pituitary cell as an intact protein composed of both subunits. Protein sequence determination by elucidation of full-length cDNA sequences in fish research really began with the pituitary hormones (growth hormone [GH], prolactin [PRL], and then FSH and LH). Characterizing the first two fish GtHs took an enormous amount of concentrated effort before gene cloning and DNA and mRNA amplification techniques were widely used. The characterization of fish GtHs was a major endeavor in fish endocrinology for over a decade with many laboratories around the world working to determine if there were one or two GtHs with disparate functions as in the mammalian system. In 1988, a series of papers describing the purification and full-length amino acid sequence of two GtH beta subunits in chum salmon (*Oncorhynchus keta*), using largely chromatographic techniques, were published (Itoh et al. 1988; Suzuki et al. 1988a, 1988b). This was followed the next year by a paper reporting the full-length cDNA nucleotide sequence of the same beta subunits in the same species (Sekine et al. 1989). It is worth noting that both efforts were made by the same group of scientists from the same laboratory. The cDNA sequence for one of the GtH beta subunits had been reported 2 years earlier (Trinh et al. 1986). Nowadays, it is rare to find instances where amino acid sequences for large peptide hormones or receptors are not initially deduced from nucleotide sequences (see Yaron et al. 2003). As an example, fish activin sequences have all been deduced by cDNA sequencing (e.g., Ge et al. 1993; Tada et al. 1998). Sequences for the small decapeptide GnRHs, on the other hand, have been almost exclusively derived by a combination of peptide degradation and mass spectrometry (see Lethimonier et al. 2004).

Although the use of molecular techniques has largely replaced biochemical techniques as a starting place for structure determination, not all structure-related questions can be answered by gene sequencing. A prime example is posttranslational processing such a glycosylation that is so critical to GtH function. The degree of glycosylation and oligosaccharide structure greatly affects the biological potency of the GtHs. That being said, molecular techniques are being used to address issues related to glycosylation. Examples include in vitro GtH expression systems with different posttranslational processing capabilities. Here, the processes of glycosylation together with the production of materials for investigating the effects of different degrees of glycosylation on GtH activity are achieved (e.g., Kamei et al. 2003; Kobayashi et al. 2003; Vischer et al. 2003; Kasuto and Levavi-Sivan 2005; Aizen et al. 2007a; Zmora et al. 2007).

Purified GtHs are not currently commercially available. A partially purified preparation of salmon GtH, SGG-100, was commercially available for a short period but no longer seem to be in use. Small quantities of the GtHs have been purified using chromatography procedures by several researchers. The limited hormone was used for various studies including establishing RIAs for measuring the GtHs (e.g., Swanson et al. 1989; Prat et al. 1996; Govoroun et al. 1998). Quantities were not great enough to allow for widespread use much less commercialization of assays. As mentioned, recombinant fish GtHs have also been synthesized; however, the expression systems required for the production of properly processed hormone are not good for high yield. Nonetheless, the greater availability of recombinant GtHs has led to development of additional RIAs and ELISAs as well as bioactive products for research (e.g., Aizen et al. 2007b). A particularly interesting approach to the production of recombinant fish GtHs was the use of transgenic rainbow trout to make recombinant goldfish GtHs (Morita et al. 2004). Once problems of increasing yield are met, there is every expectation that commercial production of fully bioactive recombinant hormone and recombinant hormone-based GtH assays will become available as they have for a select group of fish GHs and IGFs (see Novozymes-GroPep Adelaide AU).

Currently, due to the limited availability of suitable RIAs and ELISAs, much of what is being learned of GtH regulation comes from the study of GtH subunit transcription. Perhaps the widest felt contribution of molecular biology to the study of GtH regulation is its provision of this end point for any species of fish with relative ease. Regulation of the GtH subunit genes in tilapia has been reviewed and served as a good example of such investigations (Yaron et al. 2001). This end point has been used to identify association of expression of the two GtH beta subunits with reproductive stages to suggest when each of the two GtHs is functioning. Timing of expression is important since the GtHs are expected to have disparate actions and may even have opposing actions in determining which gonadal steroids are to be synthesized. The aquaculturist therefore will want to know which action of GtH needs to be augmented and when. GtH subunit expression has been used to identify regulators of the expression of GtH genes including hypothalamic peptides such as GnRHs, GnRH analogs and antagonists, growth hormone-releasing hormone, hypothalamic peptides associated with nutrition, sex steroids, members of the activin–inhibin system, growth factors, and stress hormones. More directly, response elements have been identified for fish GtH gene promoters and activity verified using in vitro reporter assays (e.g., Melamed et al. 2006). Changes in sensitivity to these effectors in association with reproductive stage or certain cofactors have been investigated. This information is the

first step in identifying new pharmaceuticals for use in controlling GtH production and possibly will serve as a basis for diagnostic tools to assess reproductive stage or malfunctions. Signal transduction mechanisms of these regulators have been identified, again, another source of information that might be exploited for controlling reproduction (see reviews Yaron et al. 2001, 2003).

A surprising finding from molecular approaches is the expression of all three GtH subunits in the fish gonad. The transcripts had been discovered by RT-PCR (real-time polymerase chain reaction) in the oocytes of gilthead sea bream (*Sparus aurata*; Wong and Zohar 2004) and in the ovary and testis by microarray analysis in the rainbow trout (von Schalburg et al. 2005). In the gilthead sea bream, the ovarian LHβ gene is actually driven by a different promoter than is the pituitary LHβ gene. Wong and Zohar (2004) also demonstrated that expression of the ovarian transcripts were increased by GnRH and decreased by a GnRH antagonist in vitro, and that GnRH increased LH release into the medium, supporting the concept that a functional GnRH–GtH system exists in the gonad. Previous studies had shown that GnRH is produced in the gonad (Grober et al. 1995; Lin and Peter 1996; Pati and Habibi 1998; von Schalburg et al. 1999) and affects reinitiation of oocyte meiosis, gonadal steroidogenesis (Habibi et al. 1988, 1989; Nabissi et al. 1997; Pati and Habibi 2000, 2002), apoptosis (Andreu-Vieyra and Habibi 2000), and possibly sex change (Soverchia et al. 2007). Predictably, GnRH receptors have also been demonstrated in the gonads (Habibi and Pati 1993; Yu et al. 1998; Moles et al. 2007). As with GtH, GnRH was first detected in the fish gonad as an mRNA transcript (Grober et al. 1995; Lin and Peter 1996). The dynamics and implications of this GnRH–GtH gonadal system have yet to be elucidated. It is interesting to note that Wong and Zohar (2004) estimate the concentrations of GtHs produced by the gonad may exceed those produced by the pituitary at certain times of the reproductive cycle.

Our discussion of pituitary hormones has concentrated on the GtHs. Most other peptide hormones of the pituitary have also been implicated in the regulation of reproduction in fish. This includes TSH, PRLs, GHs, somatolactin (SL), corticotropin, and melanotropin. However, they have not been exploited for reproductive purposes in aquaculture and therefore will not be discussed in detail. In addition, largely through molecular approaches, the activin–inhibin–follistatin system has been shown to function in fish, and activin and follistatin have been shown to act in a paracrine fashion in the fish pituitary (see review by Ge 2000). Much of what is known of the components of the activin system in fish has been derived from studies of the system in the ovary where the molecules have been shown to affect FM (Wu et al. 2000; Pang and Ge 2002; Petrino et al. 2007). Studies have confirmed that activin stimulates pituitary FSHβ expression over LHβ expression and follistatin inhibits activin activity, as in mammals (Yam et al. 1999; Yuen and Ge 2004; Cheng et al. 2007). However, there is evidence that inhibin stimulates GtH release in goldfish (Ge et al. 1992). These conserved actions of the activin system in fish suggest potential roles as regulators of GtH and therefore may be exploited as pharmaceutical manipulators of reproduction or in diagnostic tools.

The pivotal role GnRH plays in controlling reproduction, together with the proven value of GnRH-based products for controlling fish reproduction, has generated great interest in the fish GnRH system. In fish, it was shown for the first time that three GnRH forms can exist in the brains of vertebrates and reach the pituitary gland (Powell et al. 1994; Weber et al. 1997). The direct innervation of the teleost pituitary

by GnRH secreting neurons from the brain has also made the fish a favorite model for investigating the multiplicity of GnRHs, including the possibility of disparate functions. This intensive investigation of the GnRH system in fish provides many fine examples of the elegant use of molecular techniques to investigate reproductive function. Based in large part on studies in fish, it is currently thought that there are three paralogous genes for GnRH (see reviews by Parhar 2003; Okubo and Nagahama 2008). Type-I GnRHs (GnRH-I) are usually located in the preoptic area and are thought to be the primary hypophysiotropic regulators of GtHs, although types II or III may compensate if a type-I form is not present. GnRH-I includes mammalian GnRH (mGnRH) and most of the novel forms of GnRH found in fish. Type-II GnRH (GnRH-II) is usually located in the olfactory bulb and telencephalon and is chicken GnRH (cGnRH-II) in all species investigated to date. Type-III GnRH (GnRH-III) has been found predominantly in fish and is usually salmon GnRH (sGnRH) that is located in the midbrain tegmentum.

As mentioned, most known GnRH peptides in fish were sequenced for the first time using a combination of chromatography and Edman degradation or mass spectrometry. However, molecular approaches including cloning and sequencing GnRH cDNAs make it easy to determine which of these forms are present in subsequent species of interest. In addition, the small size of the GnRH decapeptides, which although it facilitated their being peptide sequenced, is in ways an impediment to their study. Specifically, their length does not provide enough sequence information for phylogenetic analysis or for the generation of specific antisera. Fortunately, GnRH is transcribed as a large precursor, prepro-GnRH. The prepro-GnRH peptides include a signal peptide (~20–25 amino acids), the active GnRH decapeptide, a processing tripeptide, and a GnRH-associated peptide (GAP; ~40–50 amino acids, see Lethimonier et al. 2004). The second prepro-GnRH, prepro-chicken-GnRH-II, was first characterized in a fish (White et al. 1994). The amino acid sequences of prepro-GnRH molecules deduced from cDNA sequences have been instrumental in elucidating the evolution of the GnRH molecules (Okubo and Aida 2001; Okubo and Nagahama 2008). In addition, the GAP portions of the prepro-GnRH molecules have been used to develop specific antisera to distinguish three forms of GnRH in the same tissue of European sea bass (González-Martínez et al. 2002, 2004; Zmora et al. 2002).

Once it was learned that multiple GnRH forms were present in the brain, immunological approaches were employed to map the location of the cell bodies and GnRH neural circuits (e.g., Okuzawa et al. 1990; Amano et al. 1991; Leprêtre et al. 1993). However, the use of in situ hybridization really opened the door to this line of investigation because this technique provides good evidence that a neuron is the source of the peptide, is at least as specific as antibody-based methods for detecting peptides, and does not require that a specific antisera be generated (e.g., Suzuki et al. 1992; White et al. 1994, 1995; Gothilf et al. 1996; Parhar et al. 1996). The elucidation of multiple prepro-GnRH sequences, together with the localization of the various GnRH forms in many species with great phylogenetic diversity, provided valuable insight into the evolution of multiple GnRH forms and functions. Changes in expression of multiple types of GnRH transcripts with different reproductive functions were soon being characterized in many species of aquacultural interest including gilthead sea bream (Gothilf et al. 1996), tilapia (Parhar et al. 1998, 2000; Soga et al. 1998), striped bass (Chow et al. 1998), European sea bass (González-Martínez et al. 2001), and barfin flounder (*Verasper moseri*; Amano et al. 2002). In addition, gene promoter regions

have been characterized as an additional molecular approach to understanding GnRH regulation (e.g., Chow et al. 1998; Kitahashi et al. 2005).

Similarly, molecular techniques have contributed to the characterization of various forms of GnRH receptor (GnRH-R) in fish. Currently there appears to be three types of GnRH-R, although only two of these types are found in fish (see reviews Millar et al. 2004; Levavi-Sivan and Avitan 2005). Both types can be found in a single species and up to five subtypes in a single species. At least five GnRH-Rs including subtypes have been identified in multiple fish species, including masu salmon (Jodo et al. 2003), European sea bass, and puffer fish (*Fugu rubripes*; Moncaut et al. 2005). The five GnRH-Rs in the masu salmon were identified using approaches similar to those described above for many GtH and GnRHs. Specifically, partial cDNAs from brain and pituitary were isolated by RT-PCR and 5′-rapid amplification of cDNA ends using primers corresponding to conserved transmembrane domains of known GnRH-R sequences (Jodo et al. 2003). The five GnRH-R sequences in the other two species were identified in the same study (Moncaut et al. 2005) taking advantage of the sequenced *F. rubipes* genome. The *F. rubipes* genome sequence database was first searched and five GnRH-R genes were identified. The corresponding cDNAs were then isolated from European sea bass. The GnRH-Rs are found throughout the body.

Tensen et al. (1997) first cloned a fish GnRH-R from African catfish (*Clarius gariepinus*). The cDNA was expressed in human embryonic kidney, 293 cells for studies demonstrating that the putative receptor activated GnRH-R signaling pathways, and the two native GnRH ligands elicited distinct signaling responses. Blomenröhr et al. (1997) also transfected mammalian cell lines with recombinant African catfish GnRH-R and mutants of the receptor to identify structures critical to receptor function. Using a similar in vitro approach, Alok et al. (2001) characterized GnRH signaling pathways by inserting recombinant striped bass GnRH-R into both a mammalian and a fish cell line and monitoring signaling pathways using luciferase reporter gene assays. The goldfish was the first vertebrate in which multiple GnRH-Rs were cloned (Illing et al. 1999), again demonstrating the important role fish have played in GnRH research in vertebrates. In this same study, in situ hybridization was used to characterize the expression pattern of the receptors, and transfection of the receptor transcripts into mammalian COS cells was used to reveal ligand selectivity, providing another example of the prominent role of molecular techniques in understanding the GnRH system.

Recent research on GnRH in fish provides good examples of how molecular techniques have facilitated our ability to look at gene expression in a single cell. The ability to investigate single cells is critical for complex tissues such as the brain where a limited number of neurons with different functions are intermingled. Parhar et al. (2003) used a micromanipulator outfitted with a micropipette to harvest single immunofluorecently labeled GnRH neurons from brain slices of tilapia (*Oreochromis niloticus*). They then used quantitative real-time PCR (qPCR) to measure GnRH mRNA content in individual cells of immature and mature male tilapia. They found GnRH mRNA to increase with maturation only in GnRH-positive cells of the preoptic area. These cells only produce a type-I GnRH-I. Interestingly, there was great variability in GnRH mRNA levels among individual cells for each GnRH form located in that particular cell. This group of researchers then moved on to use laser-captured neurons to look at transcript expression in single GnRH neurons. They investigated changes in expression of GPR54 in the different GnRH neuron types in mature and immature male tilapia and found variation in expression suggesting that expression of GPR54 might function

as a "stop signal" for neuronal migration of all three neuron types (Parhar et al. 2004). GPR54 is a member of the rhodopsin family of G-protein-coupled receptors and is the cognate receptor for kisspeptins (aka metastin; Lee et al. 1999). Together these receptors are thought to be critical in the regulation of sexual development due to their central role in regulating GnRH (see reviews by Roa and Tena-Sempere 2007; Gianetti and Seminara 2008; Roa et al. 2008). The kisspeptin–GPR54–GnRH system or KiSS1 system will be discussed in greater detail later along with regulation of puberty. In a third study, Parhar et al. turned to the target cells within the pituitary (2005). They found expression of three GnRH-Rs and observed that GnRH-R was expressed in gonadotropes, lactotropes, somatotropes, thyrotropes, corticotropes, melanotropes, and SL cells. Furthermore, GnRH-R expression profiles differed between immature and mature male tilapia for all cell types except for somatotropes and gonadotropes expressing LHβ. These findings support the direct actions of GnRH on the release of nongonadotropin pituitary hormones, as previously reported in fish (Marchant et al. 1989; Kakizawa et al. 1997; Weber et al. 1997).

Improved tools such as GnRH analogs combined with blockers of negative feedback, such as in Ovaprim, together with the complement of methods currently available to assess the physiological status of the fish, has been the basis for many recent improvements in the control of spawning. The ongoing research elucidating the regulation of the GnRH–GtH system is leading to new ways to regulate GtH synthesis and release, ultimately providing finer control over gonadal development and spawning. In addition, the new knowledge provides for an increased ability to diagnose how the reproductive system might be malfunctioning in the captive fish, thus directing the adjustment of environmental conditions, husbandry techniques, and hormone therapies to overcome the various blocks to reproductive competence. In addition, molecular techniques targeting transcripts improve the transfer of lessons learned among species, particularly to new less studied species.

Control of Puberty

Puberty is defined as the period during which an animal becomes capable of reproduction. In fish, puberty is generally considered to encompass the time from the onset of spermatogenesis through first spermiation in males, and the onset of vitellogenesis through first ovulation in females (see review by Okuzawa 2002). As mentioned previously, the phylogenetic diversity, wide range of reproductive strategies, and strong environmental impact on reproductive cycles in fish, translate into great diversity in the regulation of all aspects of reproduction including puberty. This can make it difficult to precisely define what constitutes puberty for an individual species. In some captive species, it is difficult to separate dysfunction specific to puberty from problems with seasonal recrudescence. For example, there are many species that year after year arrest gonadal development following the completion of oocyte growth and must be hormonally induced to spawn (see Zohar and Mylonas 2001). It is not uncommon for captive fish to have more successful spawning events after the first event, perhaps due in some cases to having already completed maturational processes associated with puberty before the second cycle. As we gain a better understanding of the regulatory mechanisms of both puberty and seasonal recrudescence, diagnosis and treatments can be better targeted to the specific problem.

Puberty has been successfully induced in many species by hormonal treatments. Androgen treatments have been used to induce early puberty even in very immature male fish including rainbow trout (Crim and Evans 1983), grey mullet (*Mugil cephalus*; Lee and Weber 1986), African catfish (Cavaco et al. 1998), and European sea bass (Zanuy et al. 1999). Induction of puberty in females at early stages of ovarian development has been more difficult. As mentioned earlier, many fish species complete vitellogenic growth in captivity and then must be induced to complete maturation and spawn with GnRH and GtH treatments (see Zohar and Mylonas 2001). It is more difficult to spawn those species where maturation is interrupted before the initiation or completion of vitellogenesis. Work in milkfish was presented earlier as such an example. In that case, implants of GnRH combined with an androgen, methyltestosterone (MT), were most effective for bringing even female fish through gonadal growth (Lee et al. 1986). The actual role of the androgen in the females was not clear, but induction of GtH synthesis was proposed as a possibility. Direct treatment with GtH preparations was used to induce puberty in female Japanese eel starting at stages preceding vitellogenesis (Ohta et al. 1997).

A long-term in vitro culture system has been established for eel testis, which allows hormonal induction of all stages of spermatogenesis (Miura et al. 1991). This system is a remarkable tool for investigating hormonal control of puberty at the level of the gonad. Using this system it has recently been shown that FSH-stimulated spermatogenesis is mediated through androgen production (Ohta et al. 2007). Such a complete system has not been established for ovary in any fish species. Nevertheless, Lokman et al. (2003) successfully incubated previtellogenic ovarian explants from eel for up to 18 days and demonstrated a positive effect of 11-ketotestosterone and IGF-I on oocyte growth, whereas E2 was without effect. This work suggests an alternative or additional mechanism by which the androgen may enhance the effect of GnRH implants on induction of puberty in the milkfish.

The ability to complete puberty successfully is obviously important for seed stock production in all cultivars. Controlling the timing of the onset of puberty in animals that reproduce successfully or can be induced to reproduce in captivity can also be of critical importance to aquaculture. Earlier maturation would be advantageous for egg producers and selective breeding programs, particularly when the fish first matures well beyond market size. Examples of this are hybrid striped bass where the female striped bass does not mature until 4 years of age (Sullivan et al. 1997). Not only does this put constraints on the ability to hold sufficient numbers or diversity of broodstock, it hampers selective breeding rates. Since grow out of the hybrid striped bass takes 2 years, selective breeding requires maintaining the fish for 2 years after the progenies have been evaluated for performance, thus cutting potential genetic improvement rates in half. This problem could be even more severe for late maturing aquaculture species on the horizon such as bluefin tuna (*Thunnus thynnus thynnus*; Mylonas et al. 2007).

Delaying or preventing puberty also has advantages under certain circumstances. The gonads of fish can make up a large percentage of body weight. In salmonids, such as the rainbow trout (*O. mykiss*), the gonads can constitute as much as 30% of body weight. In addition, gonadal maturation is associated with reduced growth rate in many species and with decreased meat quality in salmonids, Atlantic cod (*Gadus morhua*), and European sea bass (Zanuy et al. 2001; Porter et al. 2003; Karlsen et al. 2006; Schulz et al. 2006). The rainbow trout industry in the USA is heavily based on all

female fish being grown to market in order to avoid the earlier maturation of males. But as interest in production of larger trout increases, even maturation of females at 2 years of age is a problem. The ability to delay maturation in males for two additional years and in females for 1 more year would help producers interested in marketing a larger fish. In salmon, precocious maturation of males results in a significant reduction in the portion of marketable fish due to problems with both mortality and meat quality (Schulz et al. 2006). Tilapia is another group of species where early maturation is a hindrance. Not only do these fish slow in growth as they mature, overpopulation of submarket size fish can occur (see Phelps 2006).

Seasonality is critical to reproduction in most fishes. Thus, timing of puberty is strongly influenced by environmental and nutritional factors. Photoperiod, temperature, salinity, social interactions, and feeding rate can all affect timing of puberty (see Bromage et al. 2001). In salmonids, it has been proposed that there are multiple windows associated with environmental cues, particularly photoperiod, at which the fish must determine if it will have the energetic resources to continue maturation (Thorpe et al. 1998; Thorpe 2007). It is possible to use these factors to delay or accelerate puberty to some extent in a species-specific manner. The use of photoperiod is being used or has been shown to be effective in commercial grow-out production of Atlantic salmon (*Salmo salar*) and cod to reduce early maturation (aka grilsing; Endal et al. 2000; Peterson and Harmon 2005; Davie et al. 2007). Unfortunately, environmental manipulations can only delay or advance puberty about a year in most cases.

Better methods that can initiate puberty earlier or prevent puberty completely are sought. For systems where broodstock animals are selected from the grow-out population, it would be best if the methods that prevent puberty were reversible. Understanding the physiology behind puberty increases the possibility of finding better methods to control puberty. The investigation of puberty in mullet as reviewed by Nocillado and Elizur (2008) is an excellent example of the systematic use of molecular approaches to understand regulation of puberty, particularly in a minor species where extensive genomic resources are not available. The investigators first used RT-PCR to obtain full-length cDNAs of key genes operating in the brain–pituitary–gonad (BPG) pathway (e.g., GnRHs, GPR54, and aromatases), and then they employed genome-walking PCR to isolate promoter regions (e.g., for dopamine receptor and aromatase), characterized promoter functionality using in vitro luciferase gene-based reporter assays, and employed qPCR to characterize changes in reproductive regulatory genes throughout the BPG system.

As in the case of mullet, much of the research on puberty in fish has addressed temporal activation and activities of components of the BPG axis. Included among these studies are investigations of gene expression as in the work on mullet. Developmental profiles of reproduction-related genes including GnRHs, GtHs, dopamine, kisspeptin, melatonin, sex steroids, steroidogenic enzymes, IGFs, and the various cognate receptors during the course of puberty have been generated and suggest participation. In addition, in vivo and in vitro treatments with the hormones noted have been shown to affect puberty or activate components of the BPG axis at the transcript as well as peptide (translational) level (see review by Okuzawa 2002). As mentioned, metabolic resources available to the fish appear to be critical in the decision as to whether a fish will initiate or complete puberty. Many hormones that are known to respond to changes in metabolic state and regulate the growth axis appear to integrate the metabolic and reproductive axes of puberty in mammals. These include IGF-I,

kisspeptin, leptin, ghrelin, peptide YY, and neuropeptide Y (NPY) (see review of Fernandez-Fernandez et al. 2006). It is not clear how this metabolic information is transduced to the reproductive axis, but in mammals most of these peptides increase GnRH or GtH release. Recent evidence suggests a central role of the hypothalamic KiSS1 system (Tena-Sempere 2006). Studies in fish suggest a similar situation may exist. Ghrelin, leptin, NPY, kisspeptin, and IGF-I all have been shown to affect GnRH or GtH release in fish (Danger et al. 1991; Weil et al. 1999; Peyon et al. 2001; Unniappan and Peter 2004; Filby et al. 2008).

Also as complex are actions at the gonad, recently investigators have been taking advantage of genomic approaches to identify gonadal genes altered during puberty. Bobe et al. (2004) have used gene expression profiling to identify genes critical to processes of FM and ovulation in rainbow trout. Profiling 37 target genes by qPCR, they identified a cluster of genes that increased as the follicles achieved OMC. A second cluster of genes increased as the oocytes underwent the resumption of meiosis, whereas a third cluster of genes decreased in expression over this period. Among the more interesting findings were overexpression of IGF-I and II, and transforming growth factor-β (TGFβ) family peptides. Bobe et al. (2006) then turned to a 9,152 cDNA nylon microarray and identified 310 differentially expressed genes forming three clusters, among tissues collected at late vitellogenesis, postvitellogenesis, and FM. Ninety of these genes were further analyzed by qPCR in tissues from preovulatory fish. The study identified many genes not previously associated with the ovulatory process, confirmed a decrease in E2 synthesis during this period, and also supported the similarity of the ovulatory process to an inflammatory-like reaction.

von Schalburg et al. (2005) employed a combination of suppressive subtractive hybridization (SSH) and a 3,557 cDNA salmonid microarray to investigate bimonthly changes in gene expression during ovarian development over a 5-month period leading to spawning in the rainbow trout ovary. On average more than 240 genes were considered developmentally regulated over the study period, and as previously mentioned, the study identified transcription of all GtH subunits in gonads. Most recently, Luckenbach et al. (2008) used SSH combined with qPCR to characterize changes in ovarian gene expression during the transition from primary to early secondary oocyte growth in coho salmon (*O. kisutch*). Seventeen genes found to be differentially expressed by SSH were selected based on their function for further analysis by qPCR. Twelve of these transcripts were confirmed to be differentially regulated including genes involved in zona pellucida glycoprotein synthesis, yolk incorporation and processing, which were higher in the primary oocytes, and cortical alveoli components and genes involved with lipid accumulation in the secondary oocytes. Changes in expression of additional candidate genes including FSH-receptor, anti-Müllerian hormone, and gonadal soma-derived growth factor were also identified as significantly increased in secondary oocytes by qPCR in this same study.

Although these studies have identified participants in the regulation of puberty, it is still not clear what are the more proximate or decisive signals to initiate or complete different phases of puberty in fish. There are several hypotheses for the regulation of the onset of puberty in mammals, and they all result in an activation of the GnRH system prior to the onset of puberty (see reviews by Terasawa and Fernandez 2001; Gianetti and Seminara 2008). For this reason, the recent findings surrounding the roles of kisspeptin and its cognate receptor GPR54 in regulating GnRH have created considerable excitement over what this system might offer in terms of technologies

to regulate puberty. The mammalian prepro-kisspeptin is the 145-amino acid peptide product of the KiSS1 gene that can be cleaved to smaller kisspeptins. Those kisspeptin peptides containing the C-terminus are potent GPR54 agonists (West et al. 1998; Kotani et al. 2001; Muir et al. 2001). Kisspeptin and its cognate receptor, GPR54, are critical for the onset of puberty in mammals (de Roux et al. 2003; Seminara et al. 2003; Han et al. 2005; Seminara 2005; Shahab et al. 2005) and evidence suggests that this is true for all vertebrates (see review by Roa et al. 2008). Neurons containing the KiSS1 gene and its receptor play a central role in the integration of regulatory information derived from gonadal steroids, environmental cues such as photoperiod, and metabolic factors such as leptin that lead to the activation of the BPG axis (see Roa et al. 2008).

Molecular approaches have played a dominant role in the investigation of the KiSS1 system in fish. The first studies to address this system in fish were conducted by Parharet al. (2004) as previously mentioned. In this study, they cloned the GPR54 gene in tilapia, and using laser-capture of single GnRH neurons combined with qPCR, showed for the first time in vertebrates that GPR54 expression was localized to GnRH neurons and altered with maturation stage. More recently GPR54 has been cloned in two additional aquaculture species, the grey mullet as previously mentioned (Nocillado et al. 2007), cobia (*Rachycentron canadum*; Mohamed et al. 2007), and fathead minnow (*Pimephales promelas*, Filby et al. 2008). Expression of GPR54 changed in concert with GnRH expression during the onset of puberty in each of these species. In addition, brain levels of GnRH-receptor expression were also found to increase shortly after an increase in GPR54 expression, both preceding the onset of vitellogenesis in tilapia (Martinez-Chavez et al. 2008). Interestingly in the female fathead minnow, elevated brain expression of genes involved in sex steroid signaling (*esr1*, *ar*, *cyp19a2*) was observed in advance of increased expression of GPR54 (Filby et al. 2008).

A bioinformatics approach was used to characterize the KiSS1 gene for the first time in a nonmammalian vertebrate, the zebrafish (van Aerle et al. 2008). The human kisspeptin-10 sequence was used to query the zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), tetradon (*Tetraodon nigroviridis*), sea lamprey (*Petromyzon marinus*), and medaka (*Oryzias latipes*) genomes, followed by comparison of zebrafish genomic regions with those proximal to the human and mouse. The derived putative zebrafish KiSS1 sequences were used to clone the genes from adult zebrafish brain RNA. Although high synteny was observed across vertebrate genomes for the chromosome region containing the KiSS1 locus, the KiSS1 protein, kisspeptin, was poorly conserved among teleosts except for the region coding for kisspeptin-10. The kisspeptin-10 peptide is a decapeptide and the smallest kisspeptin peptide fragment to activate the GPR54 receptor (see Roa et al. 2008).

Little is still known of the KiSS1 system in fish beyond the sequence and temporal expression data already presented, but as mentioned, evidence thus far supports actions similar to that in mammals. Both kisspeptin and GPR54 expressions have been identified in multiple tissues including gonads (Mohamed et al. 2007; Nocillado et al. 2007), expanding further the gonadal GnRH–GtH system. GPR54 expression has been shown to be responsive to photoperiod in tilapia (Martinez-Chavez et al. 2008), and KiSS1 expression has been shown to be sensitive to estrogens and to exhibit sexually dimorphic expression in medaka. Two GPR54 receptors have been identified in zebrafish with disparate tissue expression profiles and signaling pathways (Biran et al. 2008).

Perhaps the most exciting finding for aquaculturists in terms of the regulation of puberty was that kisspeptin-10, a commercially available product, that has been shown to increase expression of GnRH and GPR54 in fathead minnow brain following intraperitoneal injection (Filby et al. 2008). This evidence that systemic application of what could be a relatively low-cost commercially available product can activate the GPR54–GnRH system in fish is very promising. Also worth noting are findings in mammals suggesting the KiSS1 system is involved in the preovulatory surge in GtHs and kisspeptin can have negative feedback effects on GnRH release, suggesting potential actions in induced spawning and delayed puberty in addition to the induction of puberty (see Roa et al. 2008).

Surrogate Broodstocking

A unique way considered to address the problems of very large late maturing species is surrogate broodstocking (see Okutsu et al. 2007). In surrogate broodstocking, primordial germ cells (PGCs) of one species, such as bluefin tuna, are transplanted into a related species, such as mackerels, considered to that would be a much more manageable broodstock animal. The surrogate host mackerel would then spawn tuna eggs. Molecular and cellular techniques are contributing to this possibility. Surrogate broodstocking has been pursued in fish primarily for the purpose of producing endangered salmonid species. However, there is great potential for direct application in aquaculture as just mentioned, and also as a tool for exploring reproductive physiology. The evolution of surrogate broodstocking techniques by Takeuchi, Yashizaki, Okutsu, and colleagues also serves as a good example of how the use of molecular techniques set the foundation for an impressive and pioneering line of investigation with broad potential benefits. A first step in the process was identifying a marker for the PGCs in rainbow trout. A *vasa*-like gene was cloned and characterized and shown to be a specific marker of germ cells using whole-mount in situ hybridization (Yoshizaki et al. 2000a). Next came a means of isolating the PGCs. Part of this process involved the characterization of the *vasa*-like gene regulatory region and this information was used to produce transgenic trout carrying a green fluorescent protein (GFP) gene driven by the *vasa*-like gene regulatory region (Yoshizaki et al. 2000b). The GFP-labeled PGCs were derived from enzymatically dissociated genital ridges of hatching embryos and sorted by flow cytometry (Takeuchi et al. 2002). The GFP–PGCs were then injected into the peritoneal cavities of hatching embryos in which the donor PGCs proliferated and differentiated into mature eggs and sperm. Live fry were produced from these gametes (Takeuchi et al. 2003). The primary objective of the project on surrogate broodstocking was met when GFP–PGCs from rainbow trout (*O. mykiss*) were transplanted into masu salmon (*O. masou*) and the recipient male salmon were bred with normal rainbow trout and produced rainbow trout offspring (Takeuchi et al. 2004). In this study, the salmon produced viable trout sperm in a year, whereas it usually takes male trout 2 years to do so. Thus, the concept that an earlier maturing species can serve as a surrogate of a later maturing species and decrease the time to puberty was demonstrated.

The technique still had weaknesses. Transgenic fish are not publically accepted for release or consumption, and few of the gametes produced by the recipients were that of the donor. To overcome these problems, chimeric RNAs composed of the GFP coding

region and the 3′-untranslated regions of the *vasa* gene that increases RNA stability were generated. The long-lived chimeric RNAs were microinjected into fertilized eggs of brown trout (*S. trutta*) and masu salmon and GFP-labeled PGCs of these species were isolated from the genital ridge of the recipient embryos and transplanted into a second rainbow trout recipient embryo. GFP-labeled PGCs were observed to migrate into the genital ridge of the recipients (Yoshizaki et al. 2005). To overcome the high number of recipient gametes, donor rainbow trout spermatogonia, which were previously shown to function like PGCs in their ability to colonize the genital ridge and multiply and differentiate into male and female gametes (Okutsu et al. 2006), were intraperitoneally microinjected into newly hatched embryos of triploid sterile masu salmon. The recipient salmon were bred and produced only trout offspring, which themselves were shown to produce viable offspring (Okutsu et al. 2007). Using a combination of testicular cells containing spermatogonia as donor materials and triploid surrogates also eliminates the need for GFP-labeled donor materials, so transgenesis is also avoided. Furthermore, it was also shown that cryopreserved PGCs could be used for transplantation (Kobayashi et al. 2007), making the viability of surrogate broodstocking a step closer to being feasible for aquaculture purposes.

Gender Control

Final sexual phenotype is the result of sex determination, which encompasses primary controls influencing the course of sex differentiation, and resulting sex differentiation, which is the process of gonad development following sex determination (Devlin and Nagahama 2002). Sex determination and sex differentiation in fish have been reviewed in depth by Devlin and Nagahama (2002). The species variability in mechanisms of sex determination in fish is astounding. Systems of sex differentiation among fish species include control by multiple sex factors, simultaneous and sequential hermaphrodism, parthenogenesis, gynogenesis, and environmental sex determination. Although there are species that are gonochoristic for whom gender appears to be determined by a single chromosome, there are a few fishes that appear to have cytologically differentiated sex chromosomes (see Beardmore et al. 2001). This variation in mechanisms of sex determination and differentiation contributes to a variety of problems with gender control in aquaculture. For example, in most salmonids it is not possible to identify the sex of individuals until relatively late in the growth phase. In sequential hermaphrodites for whom one sex requires a greater body size, broodstocks of at least two ages must be maintained. The most common reasons for controlling gender are sex-specific differences in growth phenotypes and in age to maturity. Problems concerning early puberty in males of several species including salmonids, gadoids, and European sea bass have already been discussed. But sexually dimorphic growth independent of time of puberty is also common. Tilapia species in which males have much greater growth performance than females are a prime example. The need to reduce reproductive output leading to overpopulation in grow-out conditions by growing monosex populations is also a necessity for most forms of tilapia culture.

Production of monosex populations for culture has been achieved through several approaches including interspecies hybridization and water temperature in species with temperature-dependent sex determination. However, when it comes to altering sexual phenotype for aquaculture purposes, the use of exogenous steroids remains

the prevailing approach (see Beardmore et al. 2001; Piferrer 2001). This is also true for inducing early sex reversal in sequential hermaphrodites (e.g., black sea bream, *Sparus macrocephalus*; Ruan et al. 1996; dusky grouper, *Epinephelus marginatus*; Sarter et al. 2006). The androgen, 17α-MT, has been available for use in the USA under INAD (investigational new animal drugs) exemption for masculinization of tilapia, and gender manipulation in freshwater-reared salmonids, percids, and ornamental finfish, and a NADA (new animal drug application) has been submitted for a feed-based product for these uses.

Scientific evidence supports the general safety of MT-induced sex reversal if properly applied, but there remains some public apprehension (see Phelps 2006). Interestingly, although environmental risk is the reason most often cited for not using MT, not using sex reversal is arguably the more environmentally unfriendly path if a larger view is taken. The environmental impact should also take into account the wastage of feed including fish meal that would go into the production of unmarketable precocious male salmonids and the resultant nitrogenous waste unnecessarily produced during grow out. These same arguments regarding wasting limited feed supplies such as fish meal, and release of excess nutrients into the environment, apply in tilapia culture considering the fact that the males much better feed efficiencies. Furthermore, the natural androgens coming from production of male salmon that have little market value has not been quantified. Based on what is known about sex steroid production by fish and sex steroid loads from land-based farm animal excrement, it is likely that these fish release a higher masculinizing load of androgens to the environment than the MT that would have been needed to invert sex in their male parents (see Vermeirssen and Scott 1996; Hakk et al. 2005; Johnson et al. 2006). In addition, if there is a risk of water-borne androgens from fish production, the risk can be better managed in a controlled hatchery environment where MT can be more easily metabolized, stripped, or sequestered as compared to natural sex steroid production in a marine net pen, cage, or pond. Regardless of the benefits of using steroids to affect sex differentiation, development of alternative methods would be favored. Molecular approaches are contributing to a greater understanding of sex determination and differentiation which will assist in the development of such alternative approaches.

Sex steroids are considered to be proximate regulators of sex differentiation in all fish species; therefore, the use of the steroids or of methods to manipulate the regulation of endogenous steroids is reasonable approaches, regardless of the form of sex determination. Genetic basis, however, does play an important role in the approaches available (direct vs. indirect vs. gynogenesis/androgenesis). The direct method of exogenous sex steroid-induced sex differentiation, which is the application of the steroid (or aromatase inhibitor, Guiguen et al. 1999) to the individuals whose sex phenotype is to be inverted, is applicable to all forms of sex determination because genotype is not a factor. The indirect method requires a specific genotype. The indirect method involves using steroids or similar agents to sex reverse fish to the opposite gender, then using that animal to breed with animals of the individual's genetic sex to produce offspring that are all of that individual's original genetic and phenotypic sex. In order to produce an all-female population in this way the fish must be female homogametic, and in order to produce all males the fish must be male homogametic. It is possible to produce monosex populations even when the sex of interest is the heterogametic sex, but this requires the production of supermales (YY) or superfemales, and an additional generation.

All these indirect approaches include treating a mixed sex population to obtain the first generation of fish where one of the sexes has a phenotype that is opposite to its genotype. These individuals must be identified for use in the next generation. Identifying these individuals requires a generation of progeny testing, which amounts to crossing the individuals with the homogametic sex to identify those whose progenies are all of the expected sex. Sex markers would eliminate this need for progeny testing, but they are not widely available for fish despite extensive searches. It is worth noting that only 176 of the more than 1,700 species of fish cytogenetically characterized have been found to have cytogenetically distinct sex chromosomes (Devlin and Nagahama 2002). Even more complicating is the finding in rainbow trout that heteromorphic chromosomes are identifiable in males of only some populations (Thorgaard 1977, 1983). This has led to alternative approaches such as AFLPs for identification of sex-linked markers in this species (e.g., Felip et al. 2005). Gynogenesis and androgenesis also require female homogeneity and male homogeneity, respectively, and can reduce the time required to produce monosex lines by a generation because it precludes progeny testing. Detailed discussion and description of these and other methods of sex reversal has been previously presented by Piferrer (2001).

Molecular techniques have been contributing to understanding basic mechanisms of sex determination and differentiation including the search for sex-determining genes, targets for manipulation, and sex-specific markers. A male sex-determining gene *DMY* (also known as *dmrt1bY*) has been discovered for only species of one genus of fish, *Oryzias* (medaka), and not all members possess the gene (Matsuda et al. 2002, 2003, 2007; Nanda et al. 2002; Nagai et al. 2008). The only other known sex-determining gene in vertebrates is *SRY/sry* in some mammals (Matsuda et al. 2007). The approach used by Matsuda et al. (2002) to identify *DMY* was to use recombinant breakpoint analysis followed by deletion analysis of the Y chromosome of a congenic XY female to restrict the sex-determining region of the Y chromosome. Shotgun sequencing of the resulting 250 kb region predicted 27 genes of which 3 were expressed during sexual differentiation. Of these three genes, only one, *DMY*, was Y specific. Further support for *DMY* being the sex-determining gene came from medaka with natural mutations in *DMY*. A mutation resulted in truncation of *DMY* and all XY female offspring. *DMY* was also found to only be expressed in somatic cells of XY gonads, the sertoli cells. Recently, Matsuda et al. (2007) showed that overexpression of a 117-kb fragment containing *DMY* in transgenic XX genetically female medaka is able to induce testis differentiation and subsequent male development. Furthermore, overexpression of *DMY* cDNA under control of the CMV promoter also caused XX sex reversal. It is notable that *Sry* is insufficient for complete male development, as indicated by the finding that *Sry* carrying genetically female transgenic mice are sterile (Koopman et al. 1991). *DMY* in medaka, on the other hand, is sufficient to induce complete male development in XX medaka suggesting a single gene is responsible for the functional difference between the X and Y chromosome of *O. latipes* (Matsuda et al. 2007).

Although the identification of a sex-determining gene in fish is a great step forward, the gene duplication generating *DMY* was a recent event in the evolution of the genus *Oryzias* (Kondo et al. 2003; Volff et al. 2003). *DMY* is not found in congeneric species including *O. luzonensis*, *O. celebensis*, or *O. mekongensis* (Kondo et al. 2003; Tanaka et al. 2007) or other diverse species of fish including platyfish (*Xiphophorus*

maculatus), guppy (*Poecilla reticulata*), tilapia (*O. niloticus*), and zebrafish (Kondo et al. 2003; Veith et al. 2003). The limited distribution of *DMY*, together with the fact that although the *O. latipes* has an XX–XY sex-determining system (Aida 1921), other species of *Oryzias* have a ZZ–ZW system of sex determination and a different sex chromosome (Takehana et al. 2007), suggests a variety of sex-determining mechanisms and sex chromosomes have evolved even within the same genus. Such diversity makes identification of sex-specific markers for use in applications such as indirect steroid-induced sex differentiation problematic.

Technologies for the control of sex for aquaculture purposes thus far have principally been directed at altering sex differentiation. As with puberty, molecular techniques have been employed to elucidate the physiology of sex differentiation. Several high-throughput genomic approaches have been recently conducted characterizing genomic changes in reproductive tissues surrounding the onset of sex differentiation, and also in response to androgens and antiestrogens.

A series of investigations on rainbow trout by Guiguen and colleagues provides a good example of how a systematic application of such approaches can promote the development of improved technologies for aquaculture including controlling sex. Baron et al. (2005) combined qPCR of 102 candidate gene transcripts and a global analysis approach using a hierarchical cluster method to map temporal changes in expression through sex differentiation. Results of the study support a well-conserved expression profile for sex differentiation between fish and mammals. Differences were noted including overexpression of the FSHβ subunit in the female gonad during the first oocyte meiosis, and an early testis-specific expression pattern of the *pax2* gene. This work was followed up by expression profiling of the same genes in response to a masculinizing androgen treatment (11β-hydroxyandrostenedione for 3 months in feed; Baron et al. 2008) and also using microarray (Baron et al. 2007). The studies compared genetic males, genetic females, and androgen-treated genetic females. A total of 2,474 genes displayed differential temporal expression patterns in response to androgen treatment. The overall conclusion of the study was that androgen treatment causes a dysregulation of early gonadal gene expression profiles leading to an early inhibition of female development. Dedifferentiation of the granulosa cells was interpreted as a crucial step leading to masculinization in response to the androgen treatment.

More recently, Vizziano et al. (2008) of the same group used the same treatments once again but this time also included the use of an aromatase inhibitor (1,4,6-androstenedione). A total of 100 genes were measured by qPCR. The expression profile of the females treated with the aromatase inhibitor more closely matched the expression profile of the naturally maturing males than did the expression profile of the females treated with the androgen. The results suggest inhibition of estrogen synthesis may be the more physiological approach to induce masculinization. The investigators did note that some differences in expression pattern between the androgen and aromatase-inhibitor treated animals, and the naturally maturing males may be due to an absence of factors derived from the Y chromosome in the female treated fish. These and related studies provide direction for those developing sex controlling treatments, help identify markers of labile periods during which treatments are most effective, and identify new gene targets for manipulation. Ultimately, these lines of investigation should lead to more efficient and possibly steroid-free means to control sex differentiation.

Sterility

Many of the problems alleviated by delayed puberty and monosex culture can be achieved by induced sterility. A major drawback to using sterility is that grow-out animals from these stocks cannot be bred, and therefore it is necessary to maintain a separate broodstock and employ an indirect genetic selection approach to selective breeding. Induction of sterility is sought for many aquaculture purposes. As noted, production of gonads in fish can have dramatic negative effects on growth rate, feed conversion ratios, and meat quality. In addition, sterility can address issues related to germplasm protection, overpopulation, and environmental protection primarily in the form of containment of exotic, transgenic, or genetically selected animals. It is not surprising that much attention has focused on means to induce sterility. Ultimately, the ability to turn reproductive potential on and off in an individual animal would appear most advantageous, except perhaps in a situation where sterility is favored for biosecurity of germplasm protection purposes. Such a system might be achievable with transgenics (see Maclean 2003).

Although sterility can be induced in rare cases using methods such as interspecies hybridization, the prevalent solution remains triploid induction, which is based on application of temperature or pressure shock of eggs to disrupt the meiotic spindle and block extrusion of the second polar body (duplicate maternal chromosome set) following fertilization (see Dunham 2004; Hershberger and Hostuttler 2007; Rasmussen and Morrissey 2007). Even so, triploid males in most species including salmonids still produce large testis and undergo morphological changes associated with maturation including reduced growth rates and reduction in meat quality. Reports on the effects of triploidy on important performance traits such as growth have also been mixed, ranging from improved to inferior. Induction of triploidy using pressure or temperature shock in newly fertilized eggs is relatively easy and inexpensive; however, it results in substantial egg loss and is not 100% effective. Production of triploids from crossing diploids with tetraploids (interploids or genetic triploids) theoretically increases the sterilization success rate of triploidy to 100%. In addition, the stress of the chromosome (polar body) retention shock, which may contribute to some aspects of the inferior performance of induced triploids, is avoided. The drawbacks of the approach include the labor required for the precise timing of the shock, an additional tetraploid broodstock must be maintained, and selection efforts are slowed because of the extra generation required to create the tetraploid lines. More efficient and reliable, and also reversible means of inducing sterility are, therefore, being sought.

Not surprisingly, much attention has focused on the GtH–GnRH system as a potential target for inducing sterility. Several approaches have been investigated for ablating GtH or GnRH function. The principle advantage being that fertility could theoretically be restored by treatment with exogenous hormones to induce gonadal maturation and spawning so that the fish could later serve as broodstock. Viable gene knockout technologies for fish are being pursued with recent success in zebrafish, so knocking out GtH or GnRH genes may soon be an option (Alvarez et al. 2007; Doyon et al. 2008; Meng et al. 2008). It is unlikely that approaches such as vaccination against the peptides or proteins would be cost-effective or 100% reliable (see Delves and Roitt 2005).

Attempts have been made to inhibit reproductive function in fish with GnRH-antisense technologies. The use of transgenic technologies to induce sterility has been recently reviewed (Wong and van Eenennaam 2008). Support for knocking out GnRH comes from the fact that a natural deletion in the GnRH gene in the mouse results in hypogonadism and sterility (Mason et al. 1986). The first such attempt in fish involved injection of rainbow trout eggs with a recombinant vector containing antisense for Atlantic salmon-GnRH driven by a salmon-GnRH *Pab* promoter (Uzbekova et al. 2000). The antisense was not 100% complementary. Although LH and FSH levels were not altered, and sterility was not induced, salmon-GnRH mRNA levels in the brain and protein levels in the brain and pituitary were reduced. This study was the first reported effect of antisense messenger on endogenous mRNA and protein levels in a transgenic fish. More recently, Hu et al. (2007) used a fully complementary GnRH-antisense driven by a carp β-actin promoter to induce sterility in some of the male carp (*Cyprinus carpio*) expressing the antisense, but not in females. Furthermore, plasma GtH was reduced in the sterile males. It was noted that puberty was delayed by 1 or 2 years in some of the antisense-positive females, which may have value in itself.

Targeting GnRH neuronal migration might also have potential to induce sterility. During early embryonic development, GnRH neurons migrate to their final location and also must send out fibers to reach their targets, such as the gonadotropes. If they fail to make contact with the gonadotropes because of failure to migrate or failure to develop the appropriate nerve axon tracts, the animals will be sterile, as in Kallmann's syndrome. Kallmann's syndrome, which is associated with sterility in humans, is due to a GnRH neuronal migratory defect (Schwanzel-Fukuda et al. 1989; Cadman et al. 2007). Efforts have been made to develop the capability to disrupt GnRH migration or axon terminal migration for the purpose of inducing sterility in fish. A study in medaka has shown using antisense knockdown of the *KAL-1* ortholog, a gene associated with the cause of Kallmann's syndrome, results in disruption of GnRH-I and GnRH-III neuronal migration, but also enlargement of the body cavity of the embryos (Okubo et al. 2006). Also of interest in this study was that GnRH forebrain neuronal development was analyzed by using transgenic medaka that expressed GFP under the control of the GnRH-I and GnRH-III promoters. Fertility of the transgenic animals was not assessed. A patent has recently been issued for the use of GABA (γ-aminobutyric acid) or GABA-related pharmaceuticals to induce fertility in fish by disrupting establishment of the GnRH system during early development (US patent 7194978).

A concern regarding disruption of GnRH neural networks is its potential to disrupt many critical regulatory systems other than just reproduction, including possibly growth and osmoregulation. As mentioned earlier, GnRH is expressed in many tissues outside of the reproduction axis and has been shown to induce the release of non-GtH pituitary hormones. Gene knockdown of GnRH-II and -III with morpholinos in one-cell stage zebrafish embryos also has been shown to have detrimental effects on embryonic development (Sherwood and Wu 2005).

Once again, we see the need for a greater understanding of reproductive systems to best address the needs of the aquaculturist. As a result, one area of research that has received a great amount of attention for both the purpose of induction of sterility and basic research to understand the GnRH system, in general, has been the ontogeny of the GnRH system. Elegant models for mapping the ontogeny of the GnRH system have been built around the use of fluorescent protein expression under

the control of the various GnRH promoters (promoter–reporter expression), as in the study by Okubo et al. (2006) mentioned in the previous paragraph. Another good example is the work by Palevitch et al. (2007), where they used promoter–reporter expression analysis to simultaneously look at the ontogeny of two GnRH forms in live zebrafish embryos. Enhanced GFP was driven by a salmon-GnRH promoter, and red fluorescent protein was driven by the chicken-GnRH-II promoter. In addition to the use of GABA-related molecules, already mentioned, there are many other factors that regulate the migration of GnRH neurons (see Schwarting et al. 2007) to investigate for possible exploitation.

Clearly, there are many candidate genes for inducing sterility. Safe and environmentally benign pharmacological regulators are sought. Establishment of transgenic "gain of function/loss of function" mutants would help establish which of these genes might serve as the best targets and possibly serve as the means to achieve sterility without resorting to transgenics. The attributes of a particular method to induce sterility are dependent on its intended use. If the issue is to improve the production performance then gonadal development must be fully inhibited, whereas prevention of spawning might be acceptable for strictly containment purposes. Perhaps, the answer lies in a combination of techniques such as making only monosex tetraploids transgenic so that the transgene is only conferred to the sterile triploid for aquaculture production. In addition, if the tetraploid broodstock were to escape and breed with native fish, the offspring would be sterile.

Control of Egg Quality

The fertilized egg must contain all the nutrients and regulatory information necessary to support embryonic development through the eventual transition of resulting larvae to exogenous feeding. It is, therefore, important to know what constituents need to be in the egg to best evaluate egg quality. For aquacultural purposes, it is also important to know how the constituents come to be in the egg to best ensure or manipulate egg quality. In essence, gaining greater control over egg quality is built upon gaining a thorough understanding of how an egg is properly made, which, in turn, entails unraveling the entire process of oogenesis. Available space permits a mere scratching of the surface of a detailed discussion of egg quality. A comprehensive book, *The Fish Oocyte*, reviewing topics related to fish oocyte formation has recently been published (Babin et al. 2007b), which further addresses this topic.

Much effort has been placed on identifying the nutrients required for the egg to support embryonic and larval development, primarily by conducting biochemical comparisons of eggs from clutches with different abilities to support offspring development. Similar approaches have recently been applied to the transcriptome (e.g., Aegerter et al. 2005; Bonnet et al. 2007). The roles of GtHs and sex steroids in regulating oogenesis, folliculogenesis, ovulation, and spawning to ensure proper construction of the ovum have received much attention. More recently, various classes of growth factors have also been investigated with regard to their roles in regulating gonadal development. Less emphasis has been placed on the *in ovo* production of components required for proper construction of the egg and also stored within the egg to support embryonic and larval development. Included among the *in ovo* derived biomolecules are regulatory proteins such as hormones, growth factors, and steroids; and RNAs

including mRNAs, microRNAs, and associated RNA-binding proteins. A quality ovum is of little value without quality sperm, but sperm quality and its contribution to egg quality are not addressed in this text.

As mentioned, a first step in understanding egg quality is determining what makes up a good egg. Biochemical approaches have been used to try to identify the nutrients, ions, vitamins, hormones, and so forth that constitute a high-quality egg both at ovulation and upon fertilization. More recently, these efforts have expanded to include proteomic analyses (see Chapovetsky et al. 2007). Knoll-Gellida et al. (2006) recently conducted a proteomic profile of fully grown zebrafish follicles using one- and two-dimensional polyacrylamide-gel electrophoresis (PAGE) protein fractionation and in-gel proteolysis, followed by tandem mass spectrometry for identification of the peptides present. Sixty proteins were identified. Among those not previously reported in the ovary was an RNA-binding protein, a Sjogren syndrome antigen B homologous protein, which binds to several small cytoplasmic RNA molecules and may act to stabilize the RNAs and prevent degradation. RNA-binding proteins would seem to be of particular importance in egg quality due to the temporal divide between transcription and translation of many maternal mRNAs used for egg construction, maturation, and embryonic development.

Molecular approaches have obviously contributed to identifying the RNA complement of the ovum (see Knoll-Gellida and Babin 2007). Large-scale EST (expressed sequence tag) sequencing of cDNA libraries including ovarian tissues has been conducted for fish including zebrafish (Zeng and Gong 2002; Li et al. 2004) and salmonids (Davey et al. 2001; Rise et al. 2004). Egg or germ cell-specific genes have been identified by constructing and screening oocyte-specific cDNA libraries for novel ESTs. Examples of this approach have been conducted in rainbow trout where novel transcripts such as that of an oocyte-specific oxysterol binding protein related-protein (OORP-T) which may play a role in lipid utilization (Ramachandra et al. 2007), and what appears to be a noncoding mRNA-like transcript (RtGST-1; Qui et al. 2008) were identified. Noncoding mRNA-like transcripts play roles in biological events important to egg quality such as cell differentiation and organogenesis. The prior mentioned study of fully grown zebrafish follicles by Knoll-Gellida et al. (2006) also included SAGE (serial analysis of gene expression) profiling. This study provides the first complete sequence data set of maternal mRNA in a fish ovum at the end of oogenesis.

Identifying which of the many peptides, proteins, and transcripts in the egg that might be of importance to aquaculture is a difficult task. Considering the maternal mRNA complement, survival factors are of course critical to embryos. In addition to survival factors, growth factors are also considered to be maternal mRNAs of dire consequence to egg quality. Among the first hormone or growth factor mRNAs identified in unfertilized fish eggs were IGF-I and IGF-II (Greene and Chen 1997) in rainbow trout. Since then many maternal mRNAs that are or may be important to embryonic survival and quality have been identified in fish. Myostatins, negative regulators of muscle growth, were identified in ovulated fish eggs when the ovary was included as part of tissue panel to determine if the gene was expressed only in muscle (Rodgers et al. 2001). Many maternal and even paternal genes critical for completion of oogenesis and embryonic development in zebrafish have recently been identified by mutant screening (Pelegri 2003; Dosch et al. 2004; Wagner et al. 2004). These mutants not only may identify genes critical for egg quality but also might suggest genes that can be exploited to ensure sterility when desired.

In addition to cataloging the protein and mRNA repertoires of eggs, "omics" approaches comparing eggs of different quality have been used in a series of studies by Bobe and colleagues to identify which components might be important for egg quality, might serve as markers for egg quality, or may provide insight into the mechanisms by which environment might affect egg quality in rainbow trout. Although not examining the proteome of the egg directly, Rime et al. (2004) used a proteomic approach to identify lipovitellin II fragments as a potential marker in coelomic fluid of rainbow trout for reduced egg quality associated with postovulatory aging. Approximately, 200 spots were detected in coelomic fluid by two-dimensional PAGE followed by silver staining. Twenty-two of these spots exhibited differential expression with aging and were therefore in-gel digested and eluted for analysis by mass fingerprinting in a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF MS). Six spots that increased severalfold during aging were lipovitellin II fragments, which are yolk protein components that likely leak from atretic or otherwise structurally compromised ova.

Aegerter et al. (2005) used qPCR to look at expression profiles of 39 target genes as egg quality diminished with postovulatory aging in rainbow trout. A total of eight of these transcripts were altered by aging and seven differed between high- and low-quality eggs, including IGF-I and cathepsin-Z. A recent study by Bonnet et al. (2007) used a 9,152-cDNA microarray to show that hormone or photoperiod manipulation of ovulation in rainbow trout negatively impacted egg quality and altered the transcriptome of the ovulated eggs. Among the findings was that the abundance of *prohibitin 2* mRNA was negatively correlated with the developmental potential of the egg. Similarly, Nagler et al. (2007a) used the GRASP chip to compare maternal mRNA differences between unfertilized eggs of rainbow trout exhibiting high or low early embryonic survival (>95% or >80% embryonic cleavage at 12 hours postfertilization, respectively). A total of 104 microarray targets were greater in the high-fertility fish and only 6 in the low-fertility fish. Not all targets were known genes.

Knowing which constituents are necessary to make up a good egg is of only limited benefit to aquaculture. More important is knowing how to ensure that these constituents are present in proper amounts in fully grown eggs and, possibly, how to alter the egg makeup to improve egg quality. This requires a greater understanding of reproductive process, particularly regulatory processes.

Biochemical approaches have provided most of the information on the nutrient composition of the egg. However, molecular approaches have contributed to our understanding of how nutrients are produced by the liver and then taken up, sequestered, and processed in the egg, and then utilized by the developing embryo. A study by Clark et al. (2005) on the effect of photoperiod and temperature on egg composition in striped bass is a good example of how extensively nutrient uptake systems are affected by environment. Briefly, fish maintained under a normal photoperiod cycle initiated oocyte growth before those maintained under a static photoperiod (15 hours light, spawning daylength) regardless of whether temperature cycled normally or was maintained at 18°C (spawning temperature). Nevertheless, fish exposed to cycling temperatures accumulated yolk normally whereas those exposed to static temperatures did not. Only fish exposed to cycling temperature could be induced to spawn fertile eggs. Fish exposed to static photoperiod and temperature did not contain yolk granules in the eggs but grew unusually large eggs containing only lipid droplets as the major ooplasmic inclusions, before regressing later. Interestingly, the

eggs of the fish under static conditions appeared similar to eggs in some 3-year-old virgin striped bass that failed to spawn (Holland et al. 2000). All in all, it is clear that environment has differential effects on the accumulation and processing of two major sources of nutrients, lipids and yolk protein, and, therefore, can affect egg quality in profound ways. It is, therefore, important to understand the mechanisms by which environment affects accumulation of these nutrients so that their accumulation can be better controlled under practical aquaculture conditions.

Most of the nutrients in the egg are derived from exogenous uptake of lipids and the yolk protein precursor proteins, Vtgs. Lipoproteins (e.g., low-density lipoprotein [LDL], very low-density lipoprotein [VLDL]) and Vtgs are produced by the liver in response to E2 and released into the circulation for receptor-mediated uptake or other forms of utilization by the oocyte (see reviews by Hiramatsu et al. 2002d; Babin et al. 2007a). The production, accumulation, processing, and metabolism of oocyte lipids and yolk are a complex set of processes that is highly variable among species. For example, in white perch, biochemical and molecular studies have shown that there are three distinct Vtgs produced by the liver (Hiramatsu et al. 2002c; Reading et al. 2008), and a single Vtg receptor (VtgR) with no splice variant (so called VLDL-receptor) is present in the ovary (Hiramatsu et al. 2002a, 2003, 2004). In rainbow trout, there is one form of Vtg but 20 genes and 10 pseudogenes tandemly arranged in a single cluster that likely produce very similar, if not functionally identical, proteins (Trichet et al. 2000; Buisine et al. 2002). Although there is only a single VtgR in rainbow trout, there are two splice variants, one of which has been suggested, without empirical confirmation, to be a VLDL-receptor potentially involved in uptake of neutral lipids by growing oocytes, lipids that will be important as an energy reserve for growing embryos and larvae (Prat et al. 1998). The complete Vtgs are largely involved in delivery to oocytes of phospholipids and their precursors, such as phosphatidylcholine, which are structural lipids, to growing oocytes, embryos, and larvae (reviewed by Patiño and Sullivan 2002).

Molecular cloning led to the discovery of multiple Vtgs in fish, such as the mummichog, *Fundulus heteroclitus* (LaFleur et al. 1995a, 1995b), and dual splice variants of the VtgR in rainbow trout (Prat et al. 1998). Studies have shown the VtgR mRNA is only expressed at high levels during previtellogenesis suggesting that the VtgR is translated at early stages of oocyte development and recycled to the oocyte surface during vitellogenic growth (Perazzolo et al. 1999; Agulleiro et al. 2007). This decline in VtgR transcript expression at the onset of vitellogenesis has been suggested as a possible precocious molecular marker for the onset of recruitment into vitellogenesis (Perazzolo et al. 1999; Agulleiro et al. 2007).

The multiple Vtgs are differentially present in different fish taxa, are differentially processed within species, and have disparate functions among fishes. Three major forms of Vtg are present in the superorders Acanthopterygii and Paracanthopterygii, VtgAa, VtgAb, and VtgC (Finn and Kristoffersen 2007). The VtgAa and VtgAb forms are paralogous, whereas the VtgC form, which is truncated on its C-terminus, lacks certain signature domains of complete Vtgs (e.g., phosvitin, β′-component, C-terminal component) appears to be a remnant of an ancestral form of vertebrate Vtg. The complete forms of Vtgs are taken up by receptor (VtgR)-mediated endocytosis in clathrin-coated pits that form coated vesicles. The coated vesicles fuse with Golgian lysosomal-like multivesicular bodies at which point the Vtgs undergo limited proteolysis yielding smaller yolk proteins that are stored in yolk globules (see reviews by Hiramatsu 2002d; Babin et al. 2007a). There is evidence that some Vtgs remain intact

for a time upon incorporation into the oocyte in some species (Sawaguchi et al. 2005, 2006) and that the presence of intact Vtg in individual striped bass follicles indicates that oocyte growth is still ongoing and that such fish are not yet ready for hormonal induction of spawning (Sullivan et al. 2003).

During FM, a second proteolysis of Vtg-derived yolk proteins may occur and the degree to which the yolk proteins derived from the various forms of Vtg are degraded to amino acids is variable among Vtgs and among species (Matsubara et al. 1999, 2003; Sawaguchi et al. 2006). In Acanthopterygian species, which are mostly marine, the yolk proteins derived from VtgAa undergo extensive proteolysis yielding a pool of free amino acids that helps to osmotically drive oocyte hydration and the extensive increase in oocyte volume seen in these species, which is associated with proper acquisition of egg buoyancy appropriate for pelagic existence in seawater (see Finn and Kristoffersen 2007). The yolk proteins derived from all forms of Vtg are further degraded during embryonic development (Hartling and Kunkel 1999; Carnevali et al. 2001).

Proteolysis of the Vtgs is dependent primarily on cathepsins. Eight cysteine proteinases, cathepsins, B, C, F, H, K, L, S, and Z, have been cloned and shown to be expressed in ovarian follicles of *F. heteroclitus* alone (Fabra and Cerdà 2004). The various cathepsin transcripts display different temporal patterns of expression during follicle growth and maturation, supportive of disparate actions. However, mRNA expression is not necessarily temporally associated with enzyme activity since many cathepsins in the follicle first appear as prepro enzymes that are activated later in follicle development or during embryonic development (see review by Carnevali et al. 2006). The aspartic protease cathepsin D has been shown to be involved in the initial proteolytic cleavage within the yolk vesicles of rainbow trout, masu salmon, and sea bream (Sire et al. 1994; Brooks et al. 1997; Carnevali et al. 1999a, 1999b; Hiramatsu et al. 2002b). Cathepsins L and B have been shown to be involved in proteolytic processing of yolk proteins during FM in sea bream and barfin flounder (Carnevali et al. 1999a, 2006; Matsubara et al. 2003). Evidence suggests that the MIH activates an ATPase-dependent proton pump, causing a transient decrease in ooplasm pH, which in turn activates cathepsins L and B during FM (Fagotto 1995; Carnevali et al. 1999a, 2006; Selman et al. 2001; Matsubara et al. 2003). The use of specific enzyme inhibitors in zebrafish suggests activation of cathepsin B leads to activation of cathepsin L, which is responsible for yolk proteolysis (Carnevali et al. 2006). Cathepsins A, B, C, D, E, and L have been shown to be differentially active during embryonic development of European sea bass (Carnevali et al. 2001). Furthermore, Carnevali et al. (1999b) found cathepsin D transcript size may serve as a marker for egg quality. Mechanisms regulating the temporal regulation of transcription, translation, and activation of these enzymes are not well understood but must have critical impacts on egg quality.

The physiological mechanisms underlying accumulation of ooplasm lipids are less clear. Vtg is a lipophosphoglycoprotein and does contribute lipids to the egg. Vtg is considered to be the primary source of lipids, mainly phospholipids, in fish such as salmonids that spawn demersal eggs lacking a prominent oil droplet. However, in many species substantial quantities of neutral lipid are accumulated before and during Vtg uptake. It has been proposed that the VtgR is responsible for uptake of lipids other than the phospholipids derived from Vtg, such as neutral lipids in species with a prominent oil globule (Hiramatsu et al. 2003). The potential role of a single receptor to be primarily responsible for the uptake of most of the nutrients in an egg

creates great interest in characterizing the nature of the Vtg binding site. Site-directed mutagenesis combined with the use of a yeast two-hybrid system was used to identify minimal interaction motifs between the VtgR and the Vtg in tilapia (*O. aureus*) and support an electrostatic interaction between Vtg and its receptor (Li et al. 2003). Such an interaction is consistent with a high degree of ligand-binding promiscuity necessary for the receptor to bind multiple Vtgs and other lipoproteins.

Neutral lipids may also be derived from fatty acids taken up by the oocyte after plasma VLDL is cleaved by lipoprotein lipase (LPL) within or without the oocyte. LPL activity and mRNA have been detected in the fish ovary (Black and Skinner 1987; Kwon et al. 2001; Ibáñez et al. 2003). In situ hybridization showed the LPL mRNA localized in the follicle cells of European sea bass (Ibáñez et al. 2003). Also, a fatty acid-binding protein (FABP), FABP-11, was cloned from an ovarian cDNA library from Senegalese sole (*Solea senegalensis*; Agulleiro et al. 2007). FABPs are cytoplasmic proteins that bind long-chain fatty acids and are thought to have a role in their uptake, transport, and metabolism. Interestingly, FABP-11 gene expression correlated with an increase in atresia and the authors suggest the expression may serve as a marker for ovarian atresia, which involves extensive recovery and recycling of oocyte lipids.

As indicated in the study by Clark et al. (2005), accumulation of Vtgs and lipids borne by other lipoproteins such as LDL or VLDL is affected by the environment but it was not determined if this was only due to effects on production of lipid-bearing compounds or also due to their selective accumulation. In medaka, it has been shown that the Vtg genes are differentially responsive to E2 and their responsiveness is dependent on developmental stage and environmental conditions including temperature and photoperiod (Hiramatsu et al. 2006). Furthermore, other hormones including estrone, GH, and androgens have been shown to modify E2-induced Vtg production (van Bohemen et al. 1982; Kwon et al. 2003). The mechanisms for these interactions are not fully understood, especially for multiple forms of Vtg, but they may be mediated in part at the level of the E2 receptor. Two nuclear E2 receptor subtypes and up to two isoforms of these subtypes have been cloned in fish (ERαa, ERαb, ERβa, ERβb), and display disparate tissue distributions (Hawkins et al. 2000; Hawkins and Thomas 2004; Nagler et al. 2007b). Furthermore, Vtg production appears to be primarily mediated through only one of the receptor subtypes whose expression is also upregulated in response to E2 (Davis et al. 2007; Leaños-Castañeda and Van Der Kraak 2007). It is evident from the preceding discussion that understanding hormonal regulation of hepatic egg constituent production and its sensitivities to natural and culture environmental cues are, therefore, important to egg quality. Although this discussion has been about nutrient accumulation, it must be kept in mind that other liver-derived proteins such as zona pellucida (egg envelope) that are essential to proper egg construction are also under E2 control (see Modig et al. 2007).

In addition to the physical environment affecting the E2 system, exogenous sources of contaminant estrogenic and other endocrine disrupting compounds (EDCs) are an increasing concern. These compounds can come from both pollutants and aquaculture feeds. The feed often contains estrogens and other sex steroids from fish meal, and also phytoestrogens from plant feed stuffs. Although negative effects of EDCs on egg quality have been documented in laboratory and field studies, the extent to which they may affect egg quality in an aquaculture setting is not fully known. Fortunately for aquaculture, fish make an excellent model for the study of EDCs and therefore much has been

and will continue to be learned about their effects and mechanisms of action that can be applicable to aquaculture situations (see review by Denslow and Sepúlveda 2007).

Even less is known of the regulation of *in ovo* produced constituents than hepatic-derived constituents. The extent to which production of these components is genetically programmed or responsive to extraoocyte regulatory factors, nutritional factors, or environmental factors is largely unknown. This line of research is complicated by the fact that there is often significant temporal discord between transcription, translation, and even protein activation for many genes important to oocyte function. Examples include VtgR transcription prior to Vtg uptake, cathepsin enzyme transcription well before yolk protein processing, cyclin B transcription antecedent to MPF (maturation promoting factor) activation, and of course production and deposition in oocytes of maternal mRNAs encoding embryonic proteins, all examples of genes transcribed well before their product proteins are required. The mechanisms and factors that determine and affect when transcripts are made, stored, and activated for translation, or degraded to end translation, are largely unknown. There is good progress for some of these processes, such as discoveries about the role of polyadenylation in storage and activation of transcripts necessary for FM and early embryonic development, but these advances have occurred mostly in nonfish species or in model fish species (Katsu et al. 1997, 1999; Stitzel and Seydoux 2007). The recent discovery of the action of microRNAs in deactivating maternal mRNAs during the maternal-zygote transition has shed light on this other critical stage involving delayed translation of maternal transcripts, again, mostly in nonfish species or model fish species (Giraldez et al. 2006; Schier and Giraldez 2006). Nevertheless, 14 microRNAs and Dicer, an enzyme involved in processing microRNAs, have recently been cloned and identified in the rainbow trout embryo (Ramachandra et al. 2008).

Just as genomics and proteomics have recently begun to be used to characterize differences in the transcriptome of high- and low-quality eggs, they also have been applied to comparing follicles of different stages of ovarian development for a more global view of progressive regulatory processes leading to the repertoire of biomolecules in a high-quality ovulated ovum. An assortment of these studies has been discussed under the context of sex determination and puberty (e.g., Baron et al. 2005; von Schalburg et al. 2005; Bobe et al. 2006; Luckenbach et al. 2008). As these studies have identified genes important to the progress of their particular targeted processes, it is highly likely the proper expression of these same genes is necessary for constructing high-quality eggs.

Concluding Remarks

Control of reproduction is sought to enable the culturist to produce the highest quality gametes of the desired sex when they want them, and in the most cost-effective, efficient, environmentally, and socially acceptable way possible. Molecular approaches have participated in the advancement of procedures to control reproduction for the benefit of aquaculture. Molecular approaches contribute to advances currently being made in our understanding of basic reproductive processes, which lays the foundation upon which future practical methods for control of reproductive processes will be formed. Molecular markers have been developed for reproductive stage, sex, and gamete quality. New regulatory participants in the control of fish reproduction have been identified using molecular approaches including many not yet fully annotated ESTs.

There are many more potential applications on the horizon such as recombinant-based GtH reagents for assessing reproductive status or for direct employment in hormone therapy. Recent progress on the KiSS1 system certainly supports this as being a particularly fruitful area from which reproductive control technologies might quickly emerge. Mechanisms behind egg lipid and yolk accumulation and processing are just beginning to be unraveled using molecular methods and will undoubtedly lead to altered husbandry practices for improved egg quality. Surrogate broodstocking is certainly an intriguing possibility on the horizon that was borne of genomic science. All these areas of progress support the view that molecular technologies have strongly assisted development of aquaculture biotechnology.

Many other potential applications of molecular-based technologies will depend on social acceptance. This obviously includes the extent to which transgenic technologies will be part of the aquaculturist's toolbox. Among the most promising of these technologies are those directed at greater control of puberty or sterilization. In addition, the public view toward the use of steroids for control of sex is critical to many aquaculture industries. If the use of steroids needs to be disbanded, a greater mechanistic (and molecular) understanding of sex determination, differentiation, and puberty will be needed to help find alternative approaches to controlling sex or getting around the negative implications of not controlling sex.

Exactly how molecular approaches will most significantly contribute to the control of reproduction in the future is difficult to predict. The rapid advancement in the capabilities of molecular technologies to assist in the elucidation of basic reproductive processes is simply astounding. Just looking back at how quickly qPCR has become a workhorse in most reproductive laboratories provides a great example of how new technologies are integrated into aquaculture research and development. Microarrays are just becoming common place, but already new more comprehensive technologies for gene expression profiling are on the horizon such as massively parallel sequencing approaches based on 454 sequencing (see Torres et al. 2008). These "omics"-based approaches are quickly leading to the ability to track, analyze, and interpret data (bioinformatics) as being the limiting factor. Better methods to mine these large data sets are clearly needed. The ability to routinely and economically sequence the genome and annotate the transcriptome and even proteome of aquaculture species is on the near horizon and will greatly affect how elucidation of reproductive control mechanisms is approached. Critical technologies are still lacking for both application and research. The ability to routinely knockout genes in fishes is a clear example that may soon come to pass (see Doyon et al. 2008; Meng et al. 2008).

In addition to new technologies, innovative use of existing technologies is still a major determinant of progress. The use of the GFP gene driven by promoters for *vasa* to trace germ cells, and GnRHs to track GnRH ontology, have led to very innovative approaches to control and investigate reproductive processes. The application of global gene and protein expression profiling to experimental models in development or already exploited by less comprehensive methods holds great promise to provide insight and direction for development of aquaculture applications. Among these are long-term in vitro culture systems for testis and ovary, clearly opening the door for many critical lines of investigation to elucidate more globally the actions of regulators of reproduction (Miura et al. 1991; Lokman et al. 2003). Manipulations of reproductive processes such as those behind the separation of photoperiod and

temperature effects on egg development as conducted by Clark et al. (2005) warrants more comprehensive investigation to better understand the effects of environment on egg quality. The use of unilateral ovariectomy to perturb egg developmental processes as described by Tyler et al. (1996) in rainbow trout certainly has potential to lead to methods to increase the rate of oocyte growth in farmed fishes. With the combination of new more powerful and economical molecular-based technologies, and the innovative application of these and more well-established technologies, there are doubtlessly plenty of new markers of reproductive status to be identified and many ways to alter reproductive regulatory pathways to be discovered and exploited for the purpose of gaining better control over spawning, puberty, sex determination, and egg quality in farmed fishes. The aquaculture community can look forward to a greater understanding of reproductive physiology, better methods of evaluating reproductive status, and better tools to control reproduction from research utilizing molecular and cellular techniques.

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