Aquaculture Biotechnology

Aquaculture Biotechnology

Edited by

Garth L. Fletcher

Matthew L. Rise

WILEY-BLACKWELL

A John Wiley & Sons, Ltd., Publication

Wiley-Blackwell is an imprint of John Wiley & Sons, formed by the merger of Wiley's global Scientific, Technical and Medical business with Blackwell Publishing.

Registered office: John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at www.wiley.com/wiley-blackwell.

Library of Congress Cataloging-in-Publication Data

Aquaculture biotechnology / edited by Garth L. Fletcher, Matthew L. Rise.
p. cm.
Includes bibliographical references and index.
ISBN-13: 978-0-8138-1028-7 (hard cover : alk. paper)
ISBN-10: 0-8138-1028-0 (hard cover : alk. paper)
1. Aquacultural biotechnology. I. Fletcher, G. L. II. Rise, Matthew L.
SH136.B56A73 2012
578.76–dc23

2011028323

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

This book is published in the following electronic formats: ePDF 978070963067; Wiley Online Library 9780470963159; ePub 9780470963098; Mobi 9780470963128

Set in 9.5/12 pt Times by Aptara® Inc., New Delhi, India

1 2012

Dedication

In memory of

Kenjiro Ozato
1938–2006

This monograph on aquaculture biotechnology is dedicated to the memory of Professor Kenjiro Ozato. He was one of the first pioneers to exploit the use of gene transfer to fish as a means of altering phenotypes and, thus, helped to pave the way to greater understanding of tissue-specific gene expressions.

At the time of his passing, in 2006, Kenjiro Ozato was Emeritus Professor at Nagoya University. He retired in 2002 and became an honorary member of his former laboratory, the Laboratory of Freshwater Fish Stocks at the Bioscience and Biotechnology Center of Nagoya University, where he continued his research on medaka.

Kenjiro graduated from the Department of Zoology in the Faculty of Science at Kyoto University in 1962 and joined the Graduate School of Science to study chicken embryogenesis under the tutelage of Dr. Tokindo S. Okada. In 1967, he was awarded an Assistant Professorship at the Biological Laboratory, Yoshida College, Kyoto University. Shortly after taking up this position, he spent a period of time studying cell culture techniques under the tutelage of Dr. James D. Ebert, a renowned embryologist at the Carnegie Institute. Upon returning to Japan, he was encouraged by Dr. Tokindo S. Okada to study the developmental biology of fish. Dr. Okada was convinced that "[t]he time of fish biology will come in 15 years." At that time (1970s), this

aspect of fish biology was in its infancy. There is no question that tremendous advances have been made in molecular and developmental biology since that time. Kenjiro Ozato became a pioneer in this field.

Kenjiro established an in vitro cell culture system for fish pigment cells using goldfish and attempted to understand the molecular mechanisms associated with pigment cell transformation in the *Xiphophorus* fish-hybrid melanoma system. However, *Xiphophorus*, being viviparous, complicated in vitro analyses of the cellular mechanisms. Consequently, he shifted his attention to medaka, an oviparous fish, and developed methods to introduce foreign genes into medaka eggs in order to elucidate gene function in vivo. In 1986, he successfully demonstrated the expression of the chick δ-crystalline gene in medaka embryos (Ozato et al. 1986). This was the first direct evidence for transgene expression in fish: a demonstration that helped encourage a fledgling group of like-minded biologists to follow in his footsteps. Subsequently, Kenjiro produced a great volume of work in the field of fish developmental biology using transgenic and nuclear transfer techniques.

In 1994, Kenjiro moved to the Laboratory of Freshwater Fish Stocks at the Bioscience Centre of Nagoya University, where he began his genetic studies of Professor Hideo Tomita's collection of living natural medaka mutants. During this time, he successfully helped establish a strain of transparent medaka, "see-through medaka," within which the internal organs could be observed without the need for dissection (Wakamatsu et al. 2001). Kenjiro was also eager to advance the use the medaka as a disease model as evidenced by his final contribution to fish biology with his research on polycystic kidney disease, which was well received by researchers in a variety of medical fields (Mochizuki et al. 2005).

Kenjiro introduced medaka as an animal model for research around the world (Ozato et al. 1992). He collaborated with other investigators in assessing the influence of endocrine disrupters on wild animal taxa using medaka (Wakamatsu and Ozato 2002) and contributed to research efforts involving the use of this model for research on space exploration. He helped convene two international symposia on medaka research and was very active in encouraging and supporting Asian scientists in the development of their research programs on fish by visiting them in their own countries and welcoming them to participate in workshops at Nagoya University (Japan 1996).

Kenjiro was a kind, generous gentleman who showed true humility with regard to his many accomplishments. His considerable achievements and gentle manner earned him high regard and endearment from researchers worldwide.

Excerpted from *The Fish Biology Journal MEDAKA* (2007), Vol 11, pp 1–4, with permission.

Yuko Wakamatsu
Laboratory of Freshwater Fish Stocks, Bioscience and Biotechnology Center
Nagoya University, Nagoya, Japan

REFERENCES

Japan 1996. Japan Society for the Promotion of Science, Asian Science Seminar, Reproductive Biology and Biotechnology in Aquatic Animals-Asia '96. March 16–28, 1996, Nagoya University.

Mochizuki E, Fukuta K, Tada T, Harada T, Watanabe N, Matsuo S, Hashimoto H, Ozato K, and Wakamatsu Y. 2005. Fish mesonephric model of polycystic kidney disease in medaka (*Oryzias latipes*) pc mutant. Kidney Int. 68: 23–34.

Ozato K, Kondoh H, Inohara H, Iwamatsu T, Wakamatsu Y, and Okada TS. 1986. Production of transgenic fish: introduction and expression of chicken δ–crystalline gene in medaka embryos. Cell Differ. 19: 237–244.

Ozato K, Inoue K, and Wakamatsu Y. 1992. Medaka as a model of transgenic fish. Mol Mar Biol Biotechnol. 1: 346–354.

Wakamatsu Y, Pristyazhnyuk S, Kinoshita M, Tanaka M, and Ozato K. 2001. The see-through medaka: a fish model that is transparent throughout life. Proc Natl Acad Sci USA. 98: 10046–10050.

Wakamatsu Y and Ozato K. 2002. Medaka (*Oryzias latipes*) as a fish model for endocrine-disrupting substance testing. Environ Sci. 9: 419–426.

Contents

Preface xi
Garth L. Fletcher and Matthew L. Rise
Contributors xv

Part 1: Broodstock Improvement 1

Chapter 1 Genomic Tools for Understanding the Molecular Basis of Production-Relevant Traits in Finfish 3
Marije Booman and Matthew L. Rise

Chapter 2 Advances in Genomics and Genetics of Penaeid Shrimp 21
Javier Robalino, Robert W. Chapman, Enrique de la Vega, Nuala A. O'Leary, Danielle M. Gorbach, Zhi-Qiang Du, Max F. Rothschild, Craig L. Browdy, Gregory W. Warr, and Yannick Labreuche

Chapter 3 Genetic and Genomic Approaches to Atlantic Halibut Broodstock Management 43
Michael Reith, Darrin Reid, Debbie Martin-Robichaud, and Tillmann Benfey

Chapter 4 Prospects and Pitfalls of Clonal Fishes in the Postgenomic Era 55
Barrie D. Robison and Gary H. Thorgaard

Part 2: Molecular Cytogenetics 69

Chapter 5 Application of Fluorescence In Situ Hybridization (FISH) to Aquaculture-Related Research 71
Ruth B. Phillips

Part 3: Fish Health 81

Chapter 6 The Application of Genomics, Proteomics, and Metabolomics to Studies of Fish Health 83
Stewart C. Johnson and Laura L. Brown

Chapter 7 Antimicrobial Peptides and Their Potential as Therapeutants in Aquaculture 105
Susan E. Douglas

Chapter 8 Adaptive Immunity in Finfish: A Physiological Perspective 121
Brian Dixon and Leandro A. Becker

Part 4: Viral Pathogens and Diseases 139

Chapter 9 Structural Biology and Functional Genomics of the Shrimp White Spot Syndrome Virus and Singapore Grouper Iridovirus 141
Jinlu Wu, Zhengjun Li, and Choy L. Hew

Chapter 10 DNA Vaccines for Viral Diseases of Farmed Fish and Shellfish 153
Jo-Ann C. Leong, Kristine Romoren, and Oystein Evensen

Part 5: Embryogenesis and Stem Cells 175

Chapter 11 Egg Transcriptome, the Maternal Legacy to the Embryo 177
Juan Martin Traverso, Alexis Fostier, and Julien Bobe

Chapter 12 Application of Fish Stem Cell Technology to Aquaculture and Marine Biotechnology 193
Ten-Tsao Wong and Paul Collodi

Chapter 13 Culture of Fish Head Kidney Mononuclear Phagocytes and Muscle Satellite Cells: Valuable Models for Aquaculture Biotechnology Research 207
Frederick W. Goetz, Josep V. Planas, Mònica Díaz, Dimitar B. Iliev, and Simon MacKenzie

Chapter 14 Germ Cell Transplantation in Fish: Basic Biology and Biotechnological Applications 223
Goro Yoshizaki, Tomoyuki Okutsu, and Yutaka Takeuchi

Part 6: Gene Transfer 233

Chapter 15 Spatial and Temporal Regulation of Transgene Expression in Fish 235
Ryan MacDonald and Marc Ekker

Chapter 16 Antifreeze Protein Gene Transfer—Promises, Challenges, and Lessons from Nature 253
Garth L. Fletcher and Peter L. Davies

Chapter 17 Potential Applications of Transgenic Fish to Environmental Monitoring and Toxicology 267
Hwee Boon Grace Ng, Siew Hong Lam, Hendrian Sukardi, and Zhiyuan Gong

Chapter 18 Transgenic Tilapia for Xenotransplantation 281
James R. Wright Jr., Olga Hrytsenko, and Bill Pohajdak

Chapter 19 The Potential of Enhancing Muscle Growth in Cultured Fish through the Inhibition of Members of the Transforming Growth Factor-β Superfamily 291
Michael P. Phelps and Terence M. Bradley

Part 7: Cryopreservation 303

Chapter 20 Fish Gamete and Embryo Cryopreservation: State of the Art 305
Paz Herráez, Elsa Cabrita, and Vanesa Robles

Part 8: Environmental Considerations **319**

Chapter 21 The Potential Ecological and Genetic Impacts of Aquaculture Biotechnologies: Eco-Evolutionary Considerations for Managing the Blue Revolution 321
Darek T.R. Moreau and Ian A. Fleming

Part 9: Ethical Issues **343**

Chapter 22 Aquaculture Ethics in the Biotechnology Century 345
Lyne Létourneau

Index 355

Color plates appear between pages 222 and 223.

Preface

The culture of fish began in ancient Egypt and China several thousand years ago. However, it was not until the 1950s and 1960s that modern aquaculture for food and profit had its beginnings (Beveridge and Little 2002). Today, it is the fastest growing animal food production sector worldwide. This rapid expansion of aquaculture was, and still is, a boon to biologists as well as engineers because of its requirements for highly qualified personnel with the skill sets required to solve problems essential to the development of this important and essential food resource.

A sustainable and profitable aquaculture industry is technology and innovation driven. The rapid expansion of the industry motivated by increasing demands for product and profit required the development of appropriate low-cost feeds, efficient feeding systems, and increasingly sophisticated culture facilities on land and in the water to ensure containment and to minimize environmental contamination.

All cultured food fish and shellfish were at some point captured in the wild, and many are only now going through the process of domestication: selection of the fittest to survive, grow, and reproduce in an aquaculture environment. Not all genotypes lend themselves to survival in contained culture facilities, let alone reproduce successfully and grow at a cost-effective rate sufficient to satisfy investors. This general issue led to the first successful innovation of benefit to aquaculture: the introduction of selective crossbreeding programs for Atlantic salmon in Norway in the early 1970s (Gjedrem 1997). This program clearly demonstrated that fish, in common with other domesticated animals, had desirable traits with strong heritable components.

The first major biotechnology products to prove essential to industry were antibiotics, where they were credited with preventing a total crash of the salmon culture industry in Norway in the 1980s. This technology has, to a large extent, been superseded by the development of efficacious vaccines; the first ones being simple products consisting of inactivated bacterial cultures that eventually gave way to the use of live attenuated vaccines and more recently subunit or recombinant vaccines (Sommerset et al. 2005).

A quantum leap in interest by the scientific community to look for biotechnological ways to improve aquaculture production took place with the 1982 publication by Palmiter and colleagues, demonstrating that the addition of a few extra growth hormone genes could dramatically increase the growth rates of mice. This prompted a number of scientists to try and duplicate this success by experimenting with gene transfer in fish. However, at that time, very few fish genes were available, so most researchers had to resort to the use of the available mammalian, chicken, bacterial, and viral nucleotide sequences to build gene constructs. The only exception was the Davies, Hew, and Fletcher group, who were fortunate enough to be conducting research on fish antifreeze protein genes (Fletcher and Davies 1991). Few of these early gene transfer studies proved of value to aquaculture. However, they did serve as a "proof of concept" by showing that genes could be transferred to fish, expressed appropriately, and inherited in a stable Mendelian fashion.

In addition to the paucity of knowledge about genes in fish in the 1980s, the lack of a simple and effective method for detecting transgene integrants in tissues served as a second major bottleneck to the successful development of biotechnology tools for the benefit of aquaculture. The only reliable technique available during those early years was genomic Southern blotting; a difficult and slow procedure when one considers that there can be hundreds to thousands of samples to screen. This particular hurdle was overcome by a key technological leap in the 1980s: the invention and widespread application of PCR techniques. This revolutionized the field of molecular biology and provided an effective and essential technique to genetic engineers for the detection of transgene integrants.

The next advancement of considerable value to the development of biotech tools for aquaculture was the establishment of zebrafish as a vertebrate model in the 1990s (Grunwald and Eisen 2002). Although this development did not take place with aquaculture in mind, it did bring widespread international attention to the value of research on fish.

Once zebrafish and later the Japanese Medaka were established as important vertebrate models, all of the discoveries in molecular biology that occurred in the 1970s (e.g., reverse transcriptase, restriction enzymes, recombinant DNA, and DNA sequencing technologies), the 1980s (e.g., automated DNA sequencing), and the 1990s (e.g., DNA microarrays, cDNA, and genomic DNA libraries) were widely applied to the study of fish. These developments helped train a "critical mass" of scientists that could apply their expertise to solving problems.

Today, genome sequences are available for five model fish species (fugu, tetraodon, zebrafish, medaka, and stickleback), and genome sequencing projects for aquaculture species such as Atlantic salmon and catfish are well underway (Davidson et al. 2010; Liu 2011). These genomic technologies show great promise with regard to the discovery of genes that could alleviate aquaculture production constraints such as growth rates, disease and stress resistance, low or high temperature tolerance, and for the carnivorous species, the ability to utilize plant sourced feeds. Such discoveries could be used to modify the genome and phenotype of the animal, or facilitate the development of molecular markers for selecting broodstock with production-relevant traits.

At present, the practical application of gene biotechnologies to commercial aquaculture that bring a return on the investment in research is largely restricted to the development of vaccines, broodstock selection markers, and disease diagnostics. This is, to a large extent, due to difficulties government agencies have had in developing an acceptable process for the approval of animal food products developed by biotechnological means. Observe the fact that it took from 1994, when Aqua Bounty Technologies first met with FDA USA, until 2009 for the agency to codify the procedures required to review an application to market a growth hormone transgenic salmon product (FDA 2009). It took another year for the agency to announce that the product was safe to eat (FDA 2010). However, at the time of writing this book, the FDA has yet to approve the product for sale. Investment by the company in this product is in excess of $20 million; much of it is attributable to the lack of regulatory precedents. Therefore, it is apparent that, once the guidelines for the approval of aquaculture biotechnological products have been established, they will serve as a road map for corporate researchers to follow.

This will provide investors with the confidence that investment in such endeavors is worthwhile.

This monograph brings together the major biotechnological advances that are relevant to the enhancement of aquaculture to date. It is divided into nine parts. Part 1, consisting of four chapters, deals with genomic approaches to improving fish and shellfish broodstock. Genomics is, in essence, fundamental to identifying functional genes that can be used as markers for selective breeding programs and/or genetic modification.

Part 2 deals with cytogenetic tools for genome mapping and the localization of protein-coding genes and transgenes on fish and shellfish chromosomes.

Part 3 concentrates on fish health with chapters on the physiological aspects of adaptive immunity in fish, the application of genomics to understand the health of fish, and the discovery of fish antimicrobials that could serve as therapeutants.

Part 4 consists of two chapters. The first chapter deals with proteomics and structural biology

techniques, mass spectrometry in particular, that can be used to study the viral protein structure, function, and virus–host interactions. The second chapter outlines progress toward the development of DNA vaccines for viral diseases of farmed fish and shellfish.

Part 5 consists of four chapters on the fundamental issues concerning fish embryogenesis and stem cells that range from the egg transcriptome to germ cell transplantation.

Part 6 deals with issues pertinent to gene transfer. Despite the 30-year history behind the application of this technique to aquaculture, there have been few advances. The efficiency of gene transfer is still very low; targeted integration of single copy genes has yet to be achieved and the ability to accurately predict expression levels at the transcription and translation level is nonexistent. At present, our hopes lie in research on model fish, such as zebrafish, that could point to ways of resolving these issues. It is for this purpose that this part begins with a chapter on the regulation of transgene expression in zebrafish. The remaining four chapters present examples of the range of research that is currently being carried out using gene transfer techniques. The first chapter points to the difficulties involved in producing freeze-resistant fish, and the remaining three chapters outline progress toward the generation of zebrafish for environmental monitoring, tilapia for the production of human insulin expressing islets for xenotransplantation, and follistatin transgenic trout to understand fish muscle growth.

Part 7 deals with cryopreservation, a technique that is essential for the preservation of unique genotypes.

The monograph is completed with two chapters on environmental and ethical issues (Parts 8 and 9, respectively) that should be considered when planning to apply biotechnological advances to aquaculture.

REFERENCES

Beveridge MCM and Little DC. 2002. The history of aquaculture in traditional societies. In: Ecological Aquaculture, The evolution of the blue evolution (ed. Costa-Pierce BA). Blackwell Publishing Ltd., Oxford, UK. pp 3–29.

Davidson WS, Koop BF, Jones SJM, Iturra P, Vidal R, Maass A, Jonassen I, Lien S, and Omholt SW. 2010. Sequencing the genome of the Atlantic salmon (*Salmo salar*). Genome Biol. 11: 403.

FDA. 2009. Genetically engineered animals. http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/default.htm.

FDA. 2010. AquAdvantage Salmon. Briefing Packet. Food and Drug Administration, Centre for Veterinary Medicine, Veterinary Medicine Advisory Committee. http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.pdf.

Fletcher GL and Davies PL. 1991. Transgenic fish for aquaculture. In: Genetic Engineering, Principles and Methods (ed. Setlow J). Plenum Press, New York. Vol 13. pp 331–370.

Gjedrem T. 1997. Selective breeding to improve aquaculture production. World Aquacult. 28(1): 33–45.

Liu Z. 2011. Development of genomic resources in support of sequencing, assembly, and annotation of the catfish genome. Comp Biochem Physiol. 6 (Part D): 11–17.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, and Evans RM. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein−growth hormone fusion genes. Nature. 300: 611–615.

Sommerset I, Krossøy B, Biering E, and Frost P. 2005. Vaccines for fish in aquaculture. Exper Rev Vaccines. 4(1): 89–101.

Garth L. Fletcher
Matthew L. Rise

Contributors

Leandro A. Becker
Department of Biology
University of Waterloo
Waterloo, ON, Canada

Tillmann Benfey
Department of Biology
University of New Brunswick
Fredericton, NB, Canada

Julien Bobe
Institut National de la Recherche Agronomique
INRA
SCRIBE—Campus de Beaulieu
Rennes, France

Marije Booman
Ocean Sciences Centre
Memorial University of Newfoundland
St. John's, NL, Canada

Terence M. Bradley
Department of Fisheries
Animal and Veterinary Science
University of Rhode Island
Kingston, RI, USA

Craig L. Browdy
Novus International Inc.,
Charleston, SC, USA

Laura L. Brown
Pacific Biological Station
Department of Fisheries and Oceans
Nanaimo, BC, Canada

Elsa Cabrita
Institute of Marine Sciences of
Andalusia—ICMAN
Spanish National Research Council—CSIC
Puerto Real, Cádiz, Spain

Robert W. Chapman
Marine Resources Research Institute
South Carolina Department of Natural Resources
Charleston, SC, USA

Paul Collodi
Department of Animal Sciences
Purdue University
West Lafayette, IN, USA

Peter L. Davies
Department of Biochemistry
Queen's University
Kingston, ON, Canada

Enrique de la Vega
CI OCEANOS
Cartagena, Colombia

Mònica Díaz
Departament de Fisiología
Facultat de Biologia
Universitat de Barcelona
Barcelona, Spain

Brian Dixon
Department of Biology
University of Waterloo
Waterloo, ON, Canada

Susan E. Douglas
Institute of Marine Biosciences
National Research Council
Halifax, NS, Canada

Zhi-Qiang Du
Department of Animal Science and Center for Integrated Animal Genomics
Iowa State University
Ames, IA, USA

Marc Ekker
Centre for Advanced Research in Environmental Genomics
Department of Biology
University of Ottawa
Ottawa, ON, Canada

Oystein Evensen
Department of Basic Sciences and Aquatic Medicine
Norwegian School of Veterinary Science
Oslo, Norway

Ian A. Fleming
Ocean Sciences Centre
Memorial University of Newfoundland
St. John's, NL, Canada

Garth L. Fletcher
Ocean Sciences Centre
Memorial University of Newfoundland
St. John's, NL, Canada

Alexis Fostier
Institut National de la Recherche Agronomique INRA
SCRIBE—Campus de Beaulieu
Rennes, France

Frederick W. Goetz
School of Freshwater Sciences
University of Wisconsin-Milwaukee
Milwaukee, WI, USA

Zhiyuan Gong
Department of Biological Sciences and NUS Graduate School National University of Singapore
Singapore

Danielle M. Gorbach
Department of Animal Science and Center for Integrated Animal Genomics
Iowa State University
Ames, IA, USA

Paz Herráez
Department of Molecular Biology
Faculty of Biology
University of León
León, Spain

Choy L. Hew
Department of Biological Sciences
National University of Singapore
Singapore

Olga Hrytsenko
Department of Biology
Dalhousie University
Halifax, NS, Canada

Dimitar B. Iliev
Norwegian College of Fishery Science
University of Tromsø
Tromsø, Norway

Stewart C. Johnson
Pacific Biological Station
Department of Fisheries and Oceans
Nanaimo, BC, Canada

Yannick Labreuche
Marine Biomedicine and Environmental Sciences Center
Medical University of South Carolina
Charleston, SC, USA

IFREMER Département Lagon
Ecosystèmes et Aquaculture Durable en Nouvelle-Calédonie Station de Saint-Vincent
Noumea cedex, New Caledonia

Siew Hong Lam
Department of Biological Sciences
National University of Singapore
Singapore

Jo-Ann C. Leong
Hawai'i Institute of Marine Biology
School of Ocean and Earth Science and Technology
University of Hawaii
Kaneohe, HI, USA

Lyne Létourneau
Faculté des sciences de l'agriculture et de l'alimentation Département des sciences animals
Pavillon Paul-Comtois
Quebec, QC, Canada

Zhengjun Li
Department of Biological Sciences
National University of Singapore
Singapore

Ryan MacDonald
Centre for Advanced Research in Environmental Genomics
Department of Biology
University of Ottawa
Ottawa, ON, Canada

Simon MacKenzie
Institute of Biotechnology and Biomedicine
Universitat Autonoma de Barcelona
Barcelona, Spain

Debbie Martin-Robichaud
Fisheries and Oceans Canada
St. Andrews Biological Station
St. Andrews, NB, Canada

Darek T.R. Moreau
Ocean Sciences Centre
Memorial University of Newfoundland
St. John's, NL, Canada

Hwee Boon Grace Ng
Department of Biological Sciences and NUS Graduate School National University of Singapore
Singapore

Tomoyuki Okutsu
Japan International Research Center for Agricultural Science Tsukuba, Ibaraki, Japan

Nuala A. O'Leary
Marine Biomedicine and Environmental Sciences Center
Medical University of South Carolina
Charleston, SC, USA

Michael P. Phelps
Department of Fisheries
Animal and Veterinary Science
University of Rhode Island
Kingston, RI, USA

Ruth B. Phillips
School of Biological Sciences
Washington State University-Vancouver
Vancouver, WA, USA

Josep V. Planas
Departament de Fisiologia
Facultat de Biologia Universitat de Barcelona
Barcelona, Spain

Bill Pohajdak
Department of Biology
Dalhousie University
Halifax, NS, Canada

Darrin Reid
Institute for Marine Biosciences
National Research Council
Halifax, NS, Canada

Michael Reith
Institute for Marine Biosciences
National Research Council
Halifax, NS, Canada

Matthew L. Rise
Ocean Sciences Centre
Memorial University of Newfoundland
St. John's, NL, Canada

Javier Robalino
Facultad de Ingeniería Marítima y Ciencias del Mar
Escuela Superior Politécnica del Litoral
Prosperina, Guayaquil, Ecuador

Barrie D. Robison
Department of Biological Sciences
University of Idaho
Moscow, ID, USA

Vanesa Robles
Indegsal and Department of Molecular Biology
University of León
León, Spain

Kristine Romoren
GE Healthcare AS—Oslo
Oslo, Norway

Max F. Rothschild
Department of Animal Science and Center for Integrated Animal Genomics
Iowa State University
Ames, IA, USA

Hendrian Sukardi
Department of Biological Sciences
National University of Singapore
Singapore

Yutaka Takeuchi
Research Center for Advanced Science and Technology
Tokyo University of Marine Science and Technology
Tateyama, Chiba, Japan

Gary H. Thorgaard
School of Biological Sciences
Washington State University
Pullman, WA, USA

Juan Martin Traverso
Institut National de la Recherche Agronomique
SCRIBE—Campus de Beaulieu
Rennes, France

Gregory W. Warr
Marine Biomedicine and Environmental Sciences Center
Medical University of South Carolina
Charleston, SC, USA

Division of Molecular and Cellular Biosciences
National Science Foundation
Arlington, VA, USA

Ten-Tsao Wong
Department of Animal Sciences
Purdue University
West Lafayette, IN, USA

James R. Wright Jr.
Department of Pathology and Laboratory Medicine
University of Calgary/Alberta Health Services-Calgary Zone
Calgary Laboratory Services—Diagnostic and Scientific Centre
Calgary, Alberta, Canada

Jinlu Wu
Department of Biological Sciences
National University of Singapore
Singapore

Goro Yoshizaki
Department of Marine Biosciences
Tokyo University of Marine Science and Technology
Minato-ku, Tokyo, Japan

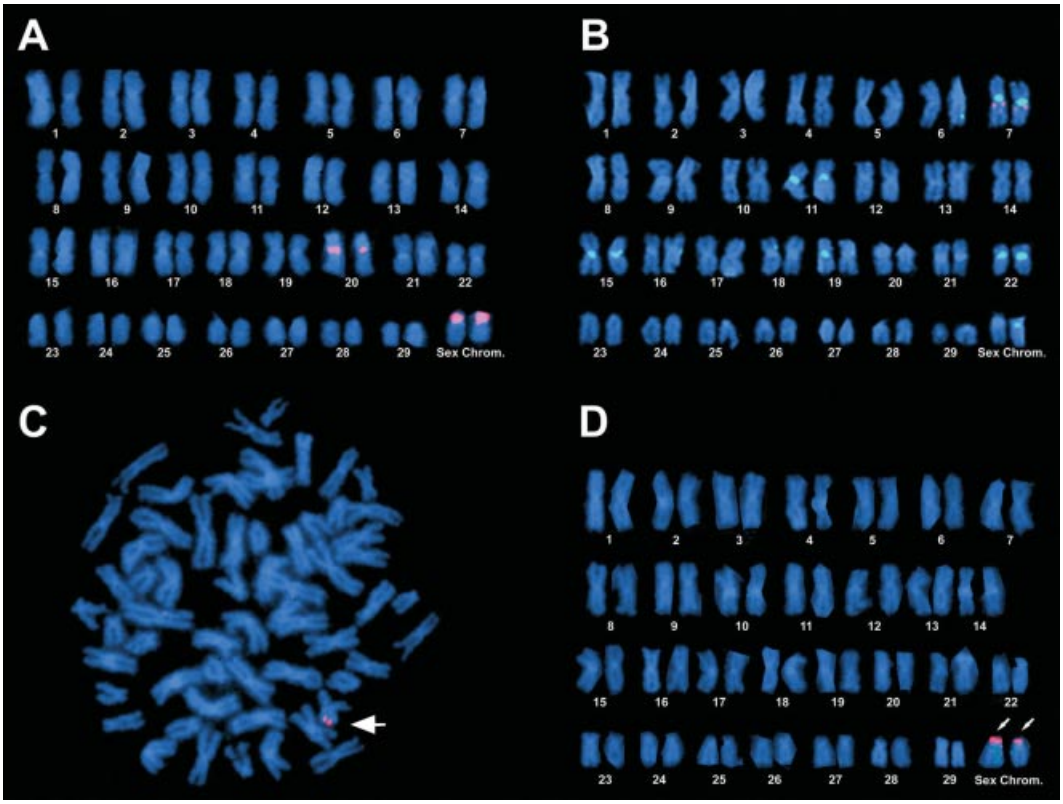


Figure 5.1. (**A**) Localization of 5S rRNA genes (labeled in red) in the rainbow trout karyotype. Note that there are two chromosomal pairs (20 and sex) with 5S rDNA with the largest amount of signal on the short arms of the X chromosomes (last chromosome pair in bottom row). (**B**) Localization of the ID1B gene to rainbow trout chromosome Omy7 (last chromosome pair in top row) using a BAC probe containing ID1B (labeled in red) and the 10H19 centromere probe (labeled in green), which is specific to several chromosome pairs including Omy7. (**C**) Localization of the growth hormone construct OnMTGH1 (labeled in red (arrow)) on the telomere of a single acrocentric chromosome in the transgenic coho strain 5750. (**D**) Identification of a YY individual in coho salmon using the GH-Y probe (labeled in red). Arrows show the location of the two Y chromosomes in this fish.

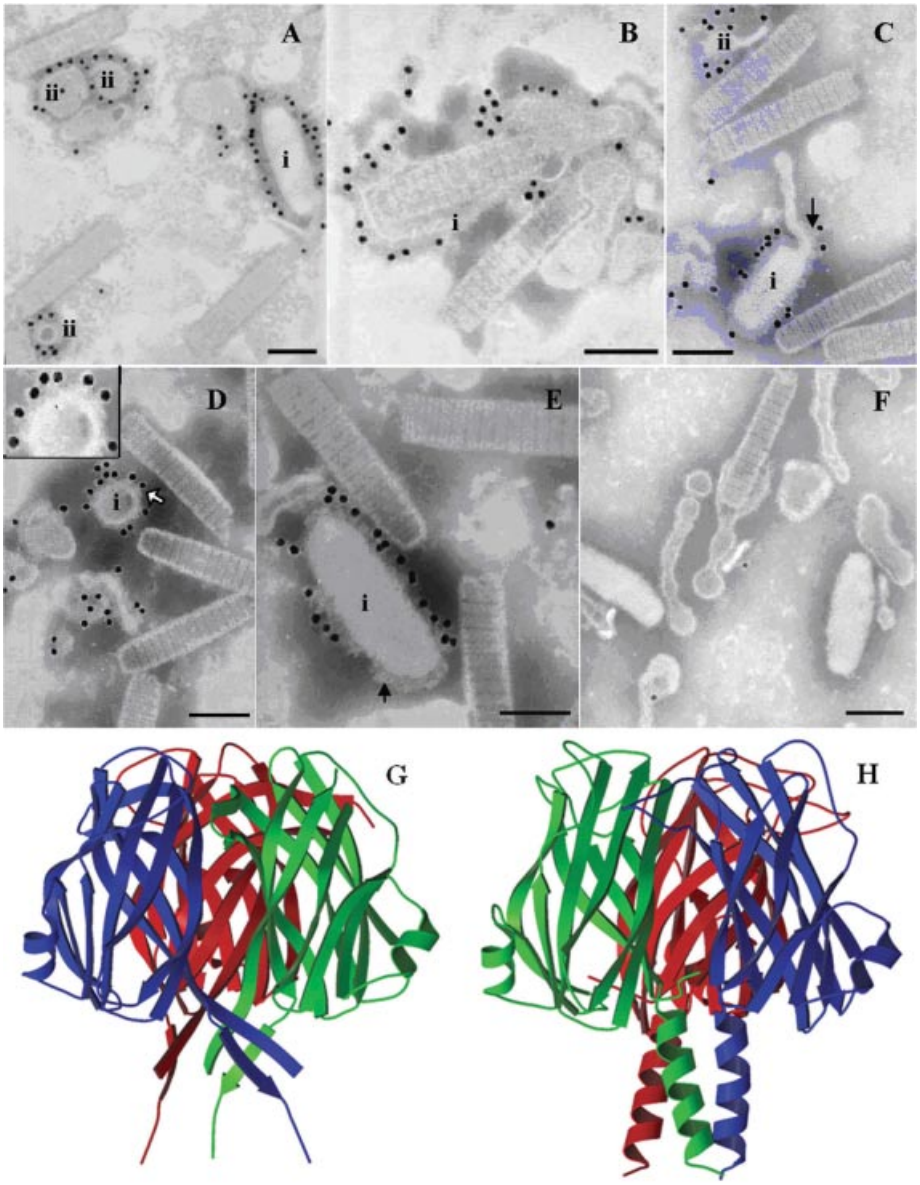


Figure 9.3. Localization and structure of WSSV envelope protein of VP26 and VP28. Purified WSSV virion was treated with 0.1% Tween 20 and then was immunogold labeled with VP26 and VP28 antibodies. Panels from **A** to **D** show VP26 antibody specifically binding to the outer surface with the gold particles localized on the outer surface of virions (**A**-i & **C**-i), semi-separated envelope (**B**-i), and completely separated envelope (envelope vacuoles, **A**-ii, **C**-ii, and **D**-i). Panel **E** shows that immunogold-labeled VP28 is also localized on the outer surface of the envelope. Surface-protruding structures (arrow) clearly appeared on some of the envelope and vesicles. The inset in panel **D** shows a large view of the surface protrusions. Panel **F** shows a very low level of nonspecific binding when a preimmune rabbit antibody or gold-conjugated secondary antibody was used. The image possibly also shows the process of vesicle formation. Panel **G** and **H** are the ribbon diagram of the VP26 and VP28, respectively. (Figures modified from Tang et al. 2007; Bar = 100 nm.)

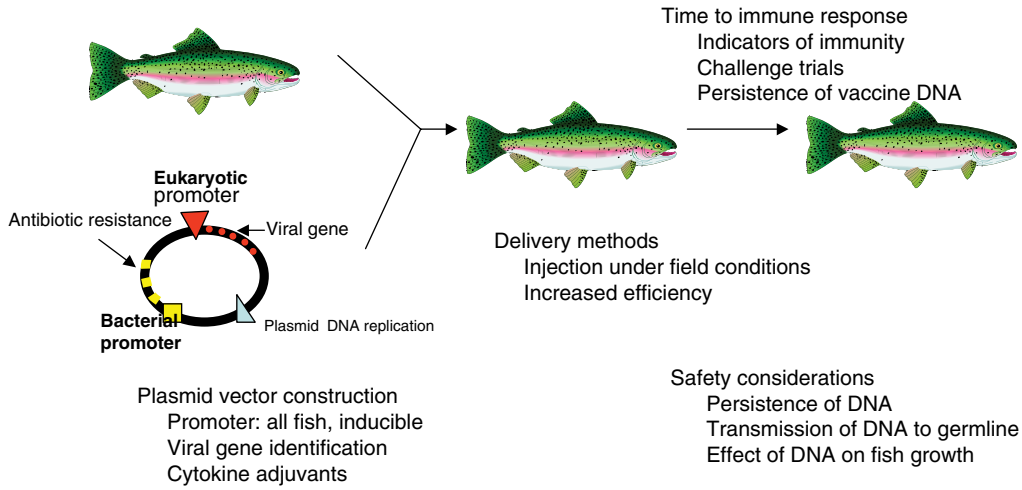


Figure 10.1. Research targets in DNA vaccine development.

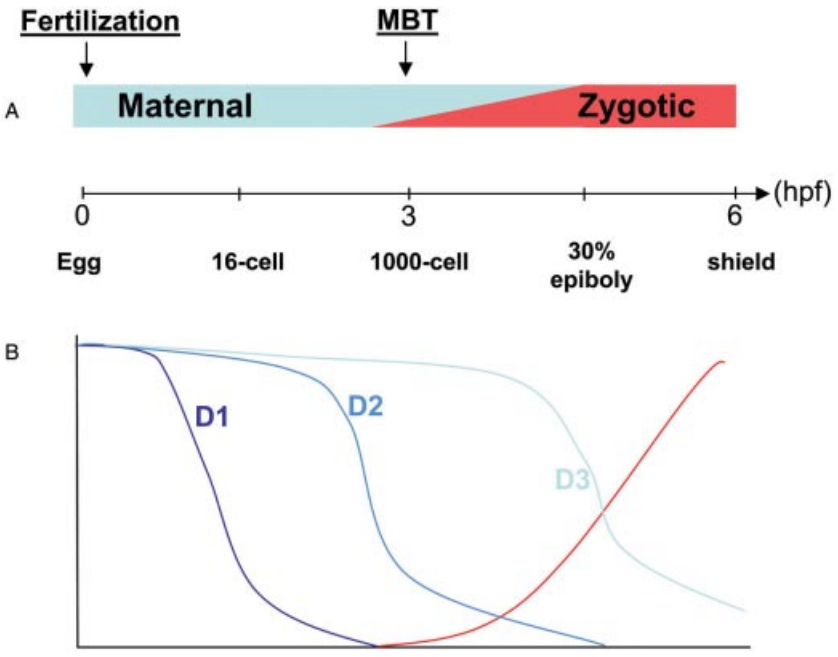


Figure 11.1. (**A**) Schematic representation of maternal and zygotic gene activities during zebrafish embryonic development. Mid-blastula transition (MBT) is indicated. (**B**) Degradation patterns of maternal mRNAs throughout embryonic development. Slow (D1), medium (D2), and fast (D3) degrading patterns are shown.

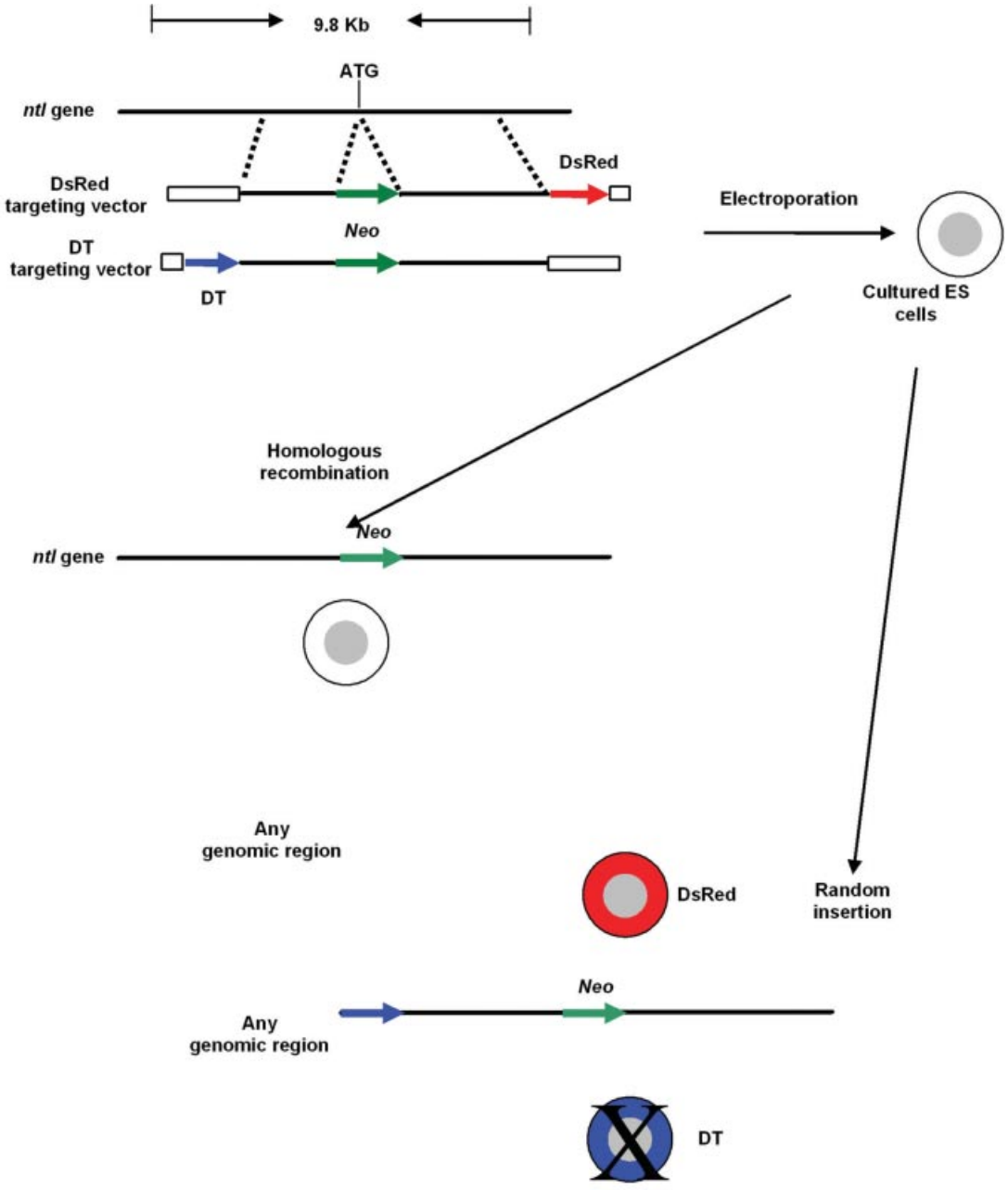


Figure 12.2. Targeted incorporation of pTG-Neo-DsRed-ntl or pTG-Neo-DT-ntl into zebrafish ES cells. The DsRed- or DT- colony is a candidate for homologous recombination.

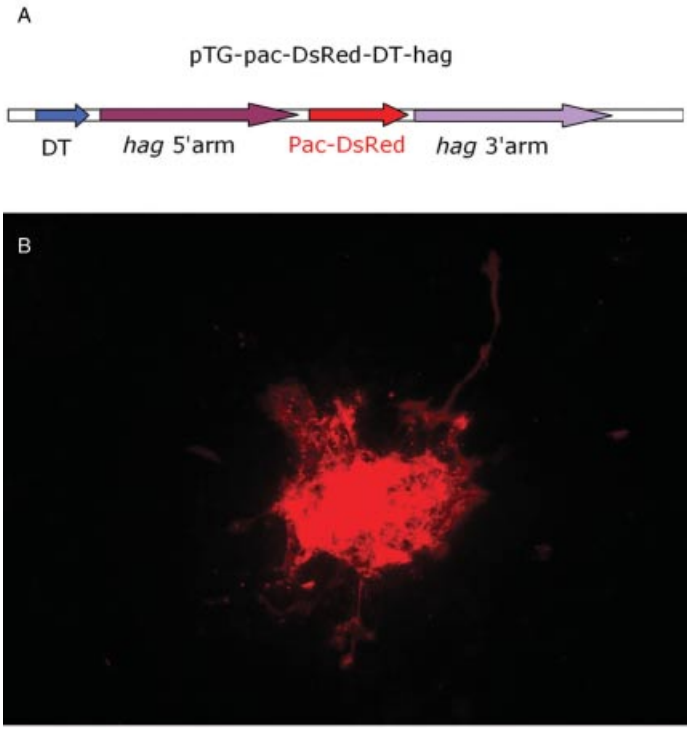


Figure 12.3. Diagram of pTG-pac-DsRed-DT-hag (**A**). In addition to puromycin (pac) selection, DsRed is used to visualize the colonies that had undergone targeted plasmid insertion by homologous recombination (**B**).

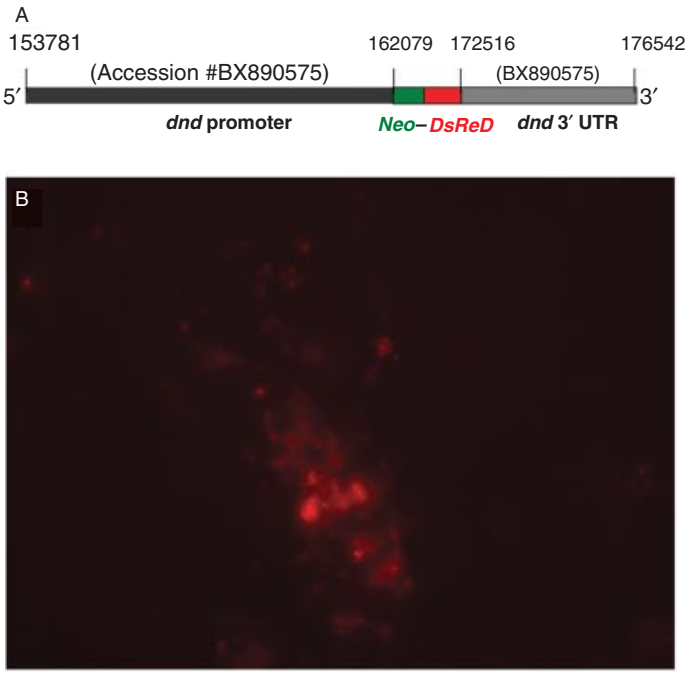


Figure 12.5. Diagram of pdnd-Neo-DsRed plasmid (**A**). The expression of Neo-DsRed fusion protein is controlled by the PGC specific promoter *dnd* and its 3′UTR. (**B**) The plasmid was injected into 1-cell embryos and later the embryos were used to initiate cultures. Following G418 selection, the DsRed expressing colonies were identified (**B**).

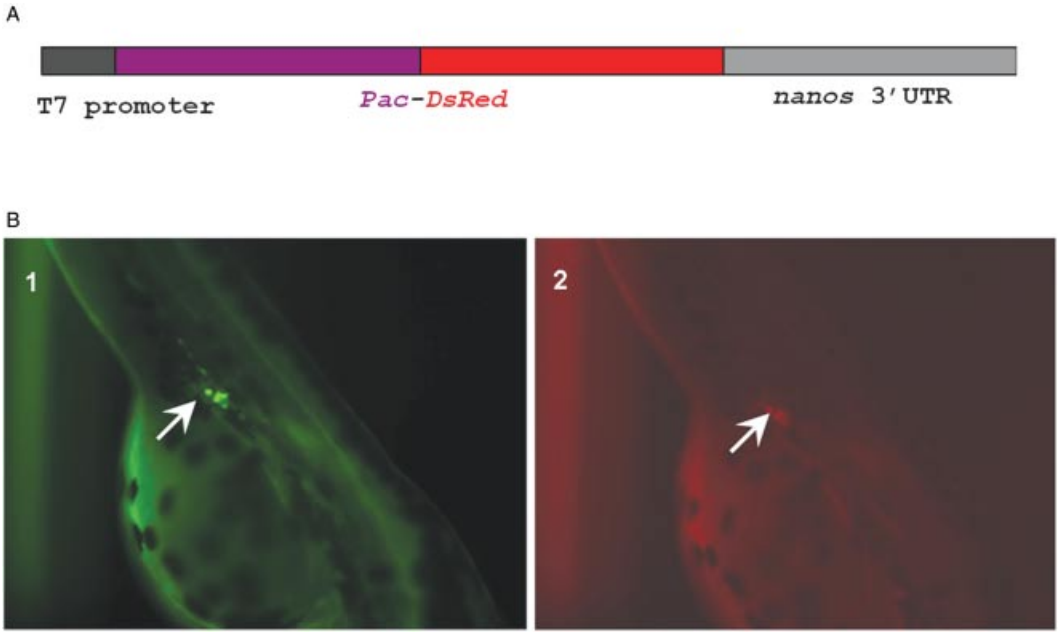


Figure 12.6. Diagram of T7-Pac-DsRed-nanos-3′UTR construct (**A**). A vasa::GFP embryo injected with *pac-DsRed-nanos*-3′UTR mRNA showing co-expression of green (1) and red (2) fluorescence specifically in PGCs (**B**). Taken from Figure 7 of Fan et al. (2008) and used with kind permission from Mary Ann Liebert, Inc.

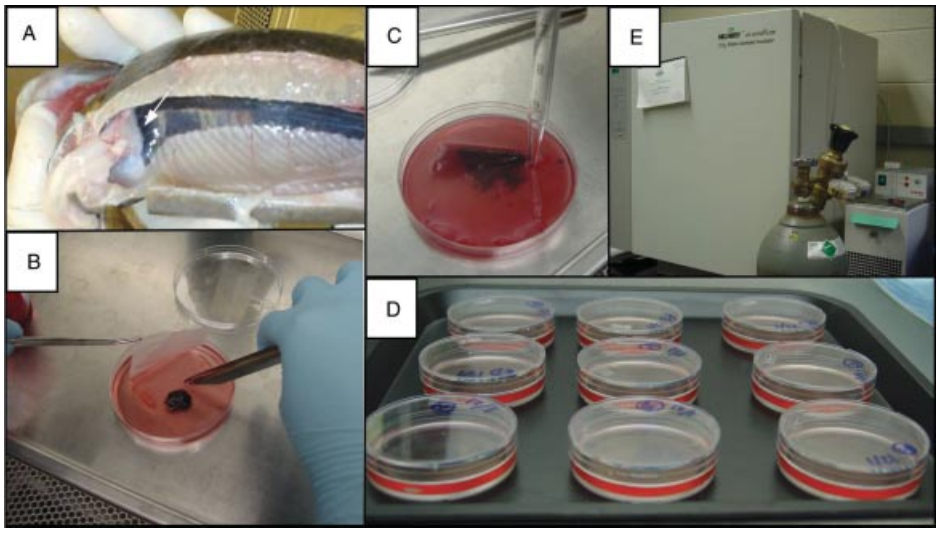


Figure 13.1. Dissection and isolation of head kidney cells from rainbow trout. (**A**) Dissected trout showing long trunk kidney with head kidney at the most anterior end (white arrow). (**B**) Head kidney placed inside of nylon mesh folder. (**C**) Pipetting dispersed cells after squeezing head kidney between nylon mesh. (**D**) Cells plated on 60 mm poly *D*-lysine treated plates. (**E**) Water-jacketed CO_2 incubator with cooling bath shown on right side.

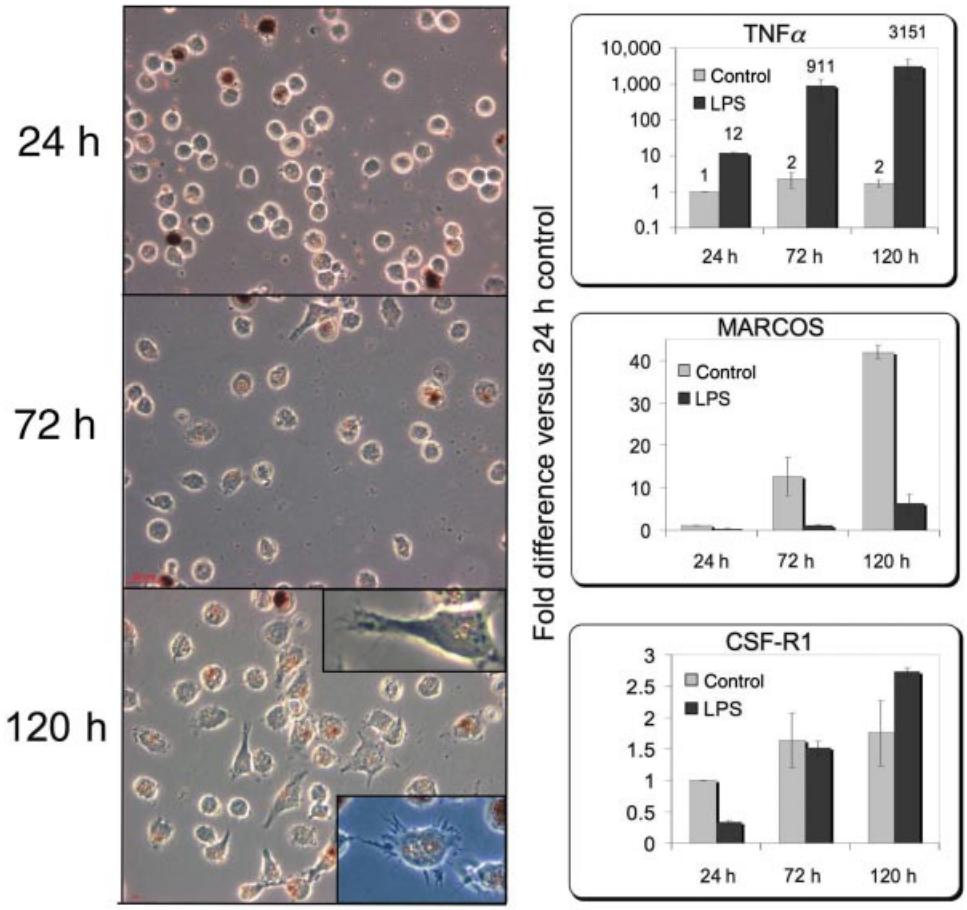


Figure 13.2. *Left*—Microscopic (360×) changes in rainbow trout mononuclear phagocytes undergoing spontaneous differentiation under in vitro conditions. Cells from head kidney homogenates were plated and allowed to adhere for 24 hours after which the nonadherent cells were removed. Inset in bottom panel is high magnification of several differentiated cells. *Right*—Comparative SYBR Green quantitative PCR analysis of the expression of tumor necrosis factor α (TNFα); MARCO; and CSF1R in trout macrophages with or without 25 μg/mL of *E. coli* LPS for 24 h and assayed at 24, 72, or 120 h. Results are shown as the mean of the fold difference of each of the samples vs. the 24 h controls $\pm$ SE ($n = 2$). Note that, the ordinate in panel **A** is logarithmic and the numbers above the histogram columns show the actual fold difference values for the respective samples.

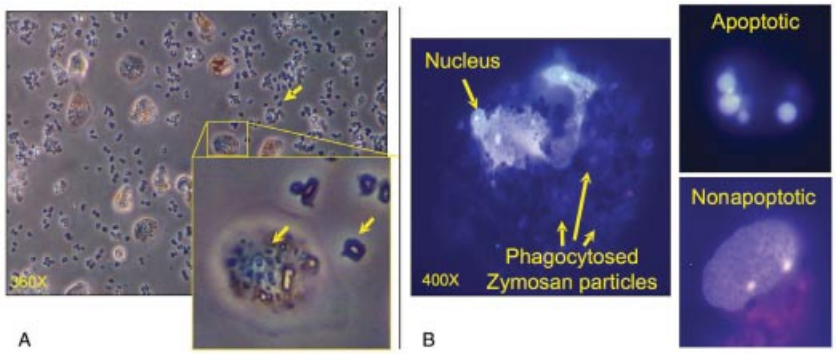


Figure 13.3. Phagocytosis of zymosan by primary trout mononuclear phagocytes. Adherent cells from trout head kidney were cultured for 5 days prior to exposure to zymosan particles. (**A**) Phase contrast imaging of cells exposed to zymosan for 30 h. Zymosan particles are indicated with arrows. (**B**) Fluorescent micrographs of trout macrophages stained with DAPI (a nucleic acid stain). Images include zymosan-exposed and control, nonapoptotic and apoptotic (treated with wortmannin, a PI3 kinase inhibitor) macrophages. Briefly, cells were fixed with 4% paraformaldehyde, washed with PBS, stained, and mounted on microscope slides. Following several washes, zymosan particles remained associated with the cells, indicating that the particles had been internalized. Comparison with the control apoptotic and nonapoptotic cells shows that although the morphology of the nucleus of the phagocytotic cell has been altered, the structure of the chromatin appears to be intact (e.g., it is still composed of clearly discernible heterochromatic and euchromatin regions in contrast with the condensed chromatin of the apoptotic cells). This demonstrates that the cells are highly phagocytic. It also suggests that, following phagocytosis of allogenic material, trout macrophages are able to survive and remain transcriptionally active for a prolonged period, a characteristic of antigen-presenting cells.

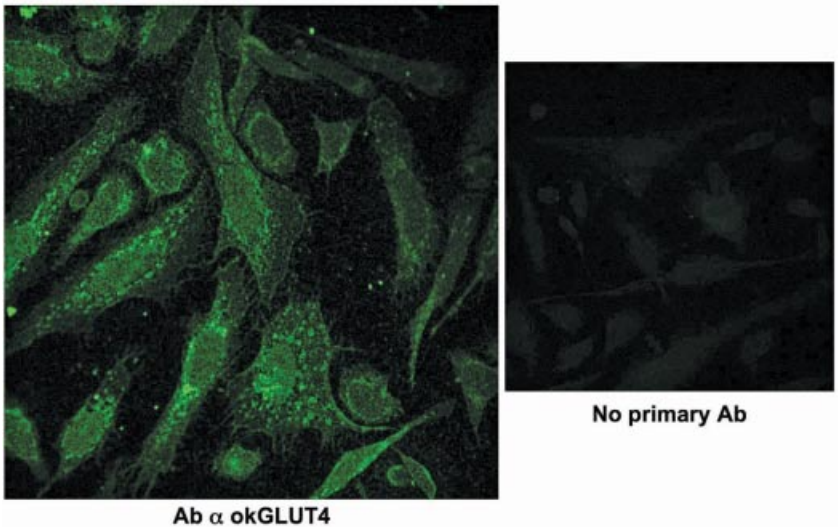


Figure 13.6. Subcellular localization of endogenous GLUT4 in trout muscle cells. (**A**). Immunofluorescence of trout GLUT4 in cells at day 5 of culture using the anti-okGLUT4 (salmon GLUT4) (Capilla et al. 2004) as primary antibody and AlexaFluor488-conjugated antibody. (**B**) Background fluorescence (in the absence of primary antibody), indicating that the staining in Fig. 13.3A was not due to a nonspecific signal from the secondary antibody.

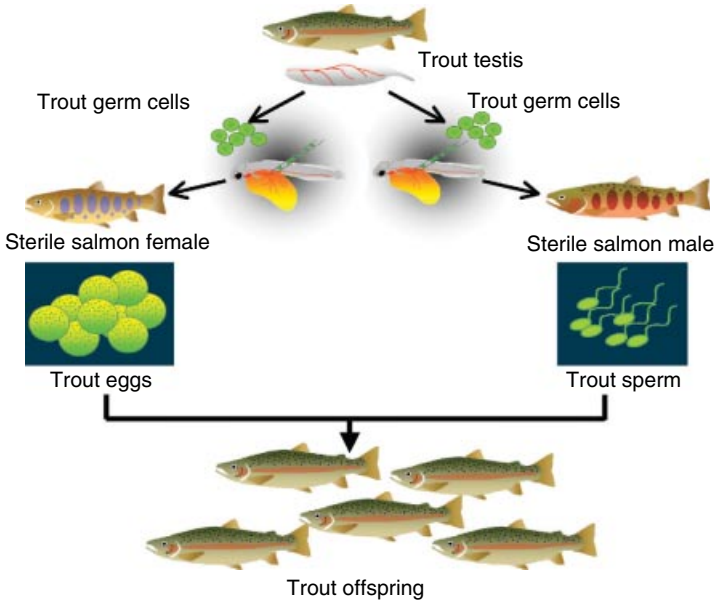


Figure 14.1. Principle for production of eggs and sperm of a different species in surrogate parents by germ cell transplantation.

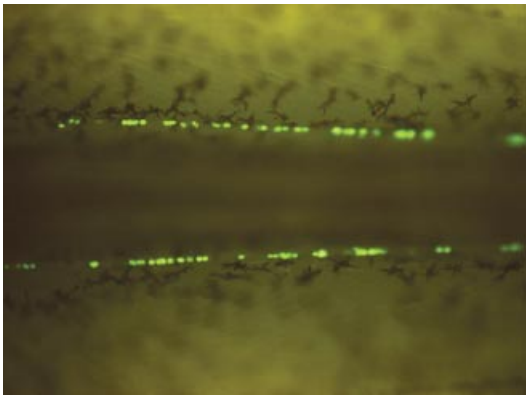


Figure 14.2. Primordial germ cells of rainbow trout transfected with the vasa-GFP gene. Photograph of the ventral side of the peritoneal cavity. The fluorescent cells aligned in two rows along the dorsal wall are primordial germ cells.

Figure 14.3. Microinjection of germ cells into the peritoneal cavity of newly hatched embryos.

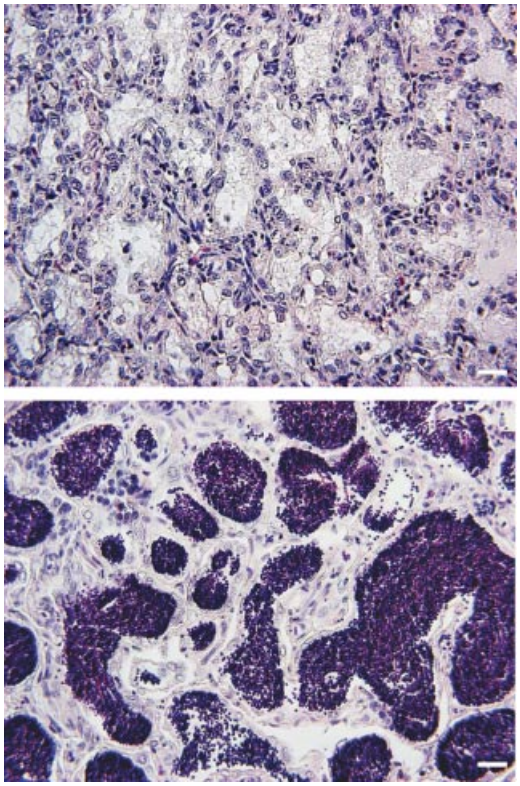


Figure 14.6. Cross sections of testis triploid masu salmon. Upper panel is of a normal triploid salmon. No sperm are present. Lower panel is of a recipient triploid salmon into which rainbow trout spermatogonia had been transplanted. Scale, 20 μm.

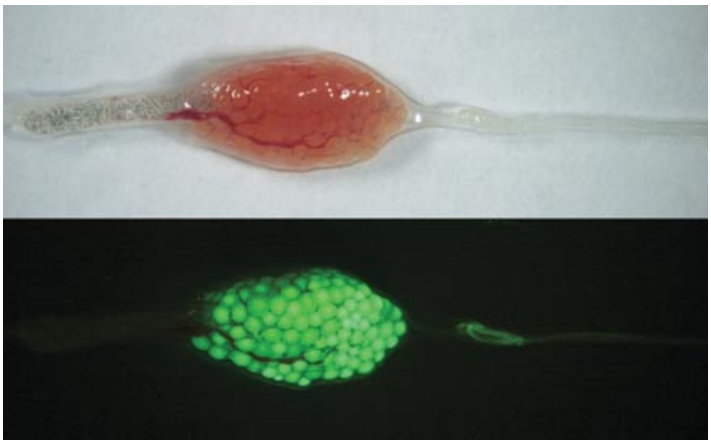


Figure 14.7. Ovaries of triploid masu salmon recipients into which rainbow trout spermatogonia had been transplanted. The top is a bright field image, and the bottom is a fluorescent field image.

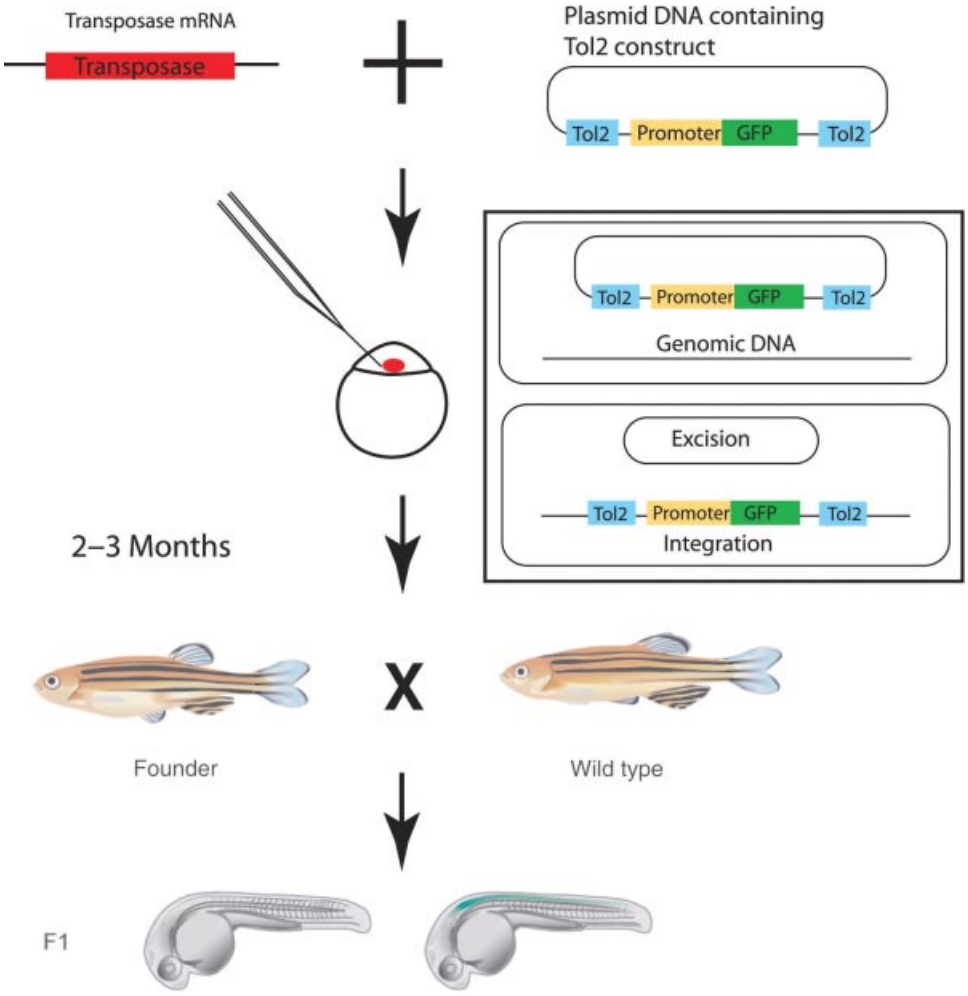


Figure 15.1. Generating transgenic zebrafish using Tol2-mediated transgenesis. The transposase mRNA is injected with the plasmid construct containing a spinal cord-specific promoter region and the reporter gene, green fluorescent protein (GFP), into fertilized eggs. The Tol2 construct will be excised from the plasmid before the promoter-GFP construct is inserted into the fish genome. The injected fish are grown to adulthood and mated for germline transmission of the transgene. The founder fish are mosaic and will have a mix of nontransgenic and hemizygous transgenic F1 progeny. (Figure modified from Kawakami 2007).

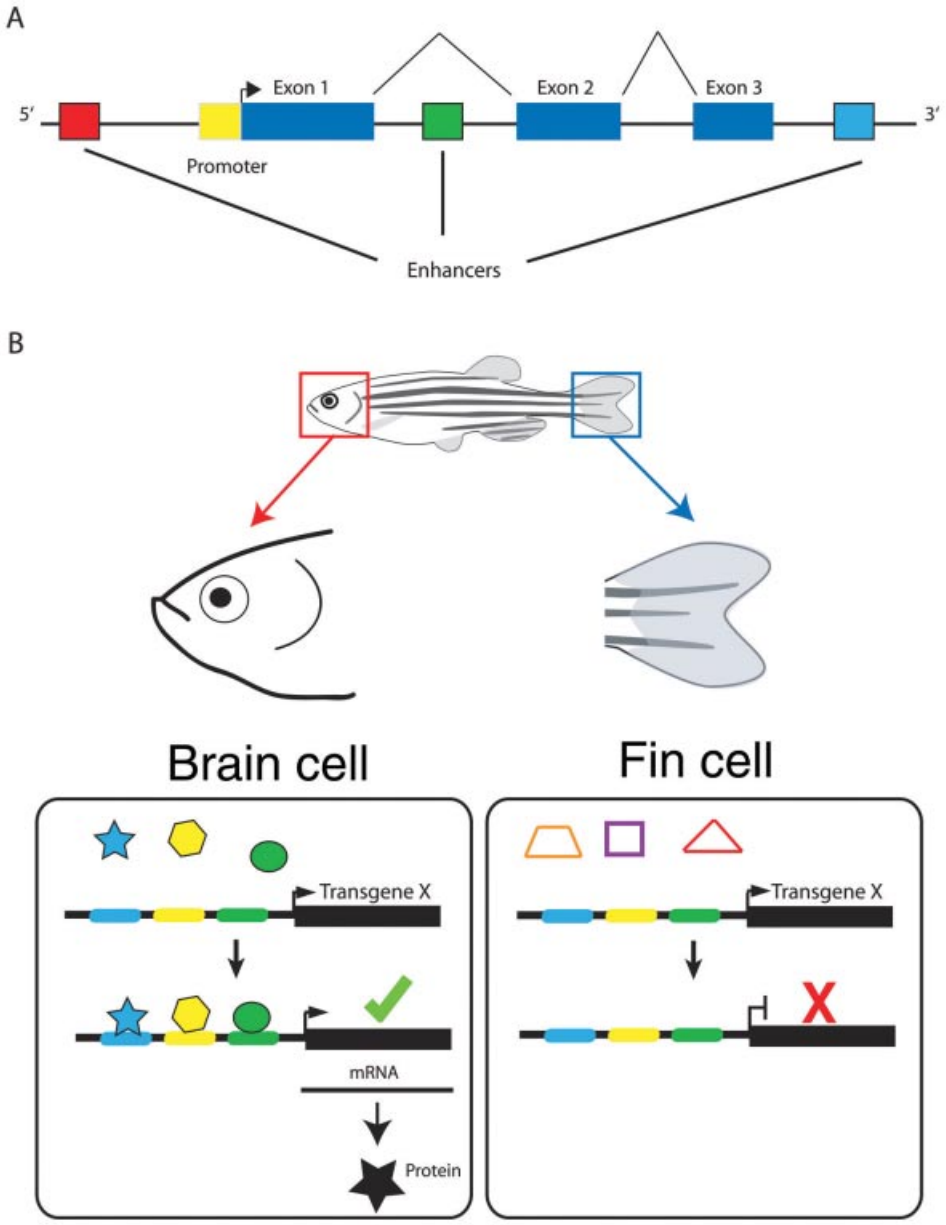


Figure 15.2. (**A**) Structure of a gene containing exons, a promoter region, and enhancers. The enhancers can be found upstream, within an intron, or downstream of the coding sequence. (**B**) Tissue-specific expression of a transgene can be obtained by generating a gene construct in which regulatory elements are controlling expression of the transgene of interest. Transcription factors will specifically bind DNA regulatory sequences based on their DNA-binding affinity preferences. In this example, the brain cells contain the proper combination of transcription factors to bind to regulatory sequences, whereas the fin cells do not. Transcription factor binding increases the frequency of transcription initiation at the promoter leading to higher mRNA and subsequent protein production within the cell.

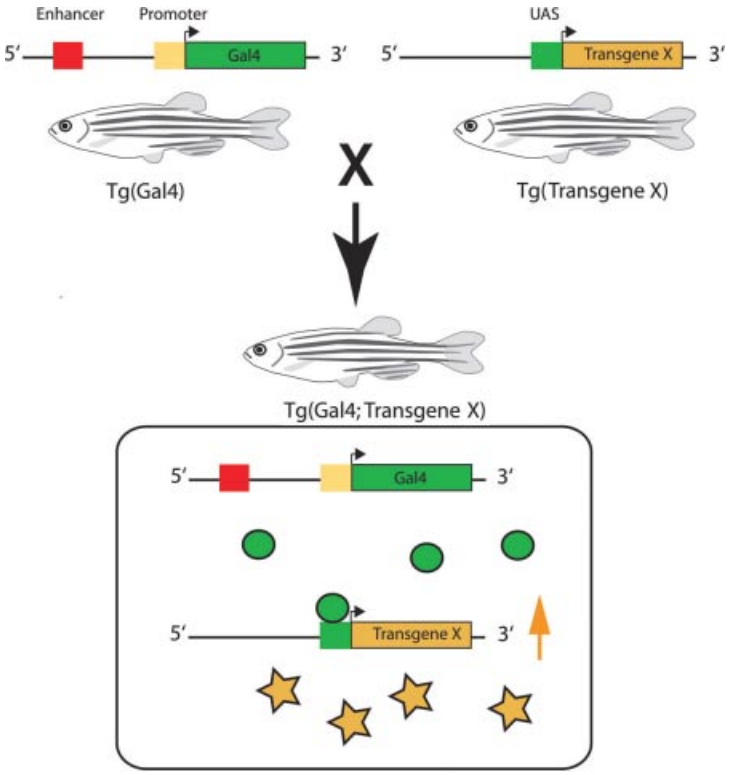


Figure 15.3. The Gal4-UAS binary system for tissue-specific expression of transgenes. The Gal4 transgene expression is controlled by tissue-specific regulatory elements (enhancer/promoter). The transgene of interest, Transgene X, is under the control of a Gal4-responsive regulatory element called upstream activating sequence (UAS). Individual transgenic fish are generated for each construct. These transgenic fish are mated to produce animals containing both constructs. The Gal4 protein (circles) will be produced in those cells where the regulatory elements are active and will bind to the UAS of Transgene X to stimulate its transcription.

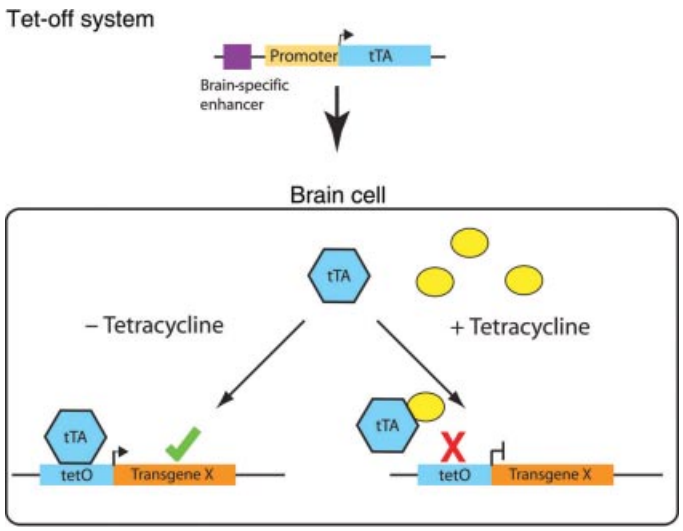


Figure 15.4. The tetracycline-off system for induction of transgene expression. Expression of the tetracycline activator, tTA, is driven by brain-specific regulatory elements. In the brain, the tTA is expressed and will bind to the tetracycline-induced promoter tetO, stimulating transcription of Transgene X. In the presence of tetracycline, the ability of the tTA to bind the tetO is blocked and Transgene X will not be transcribed.

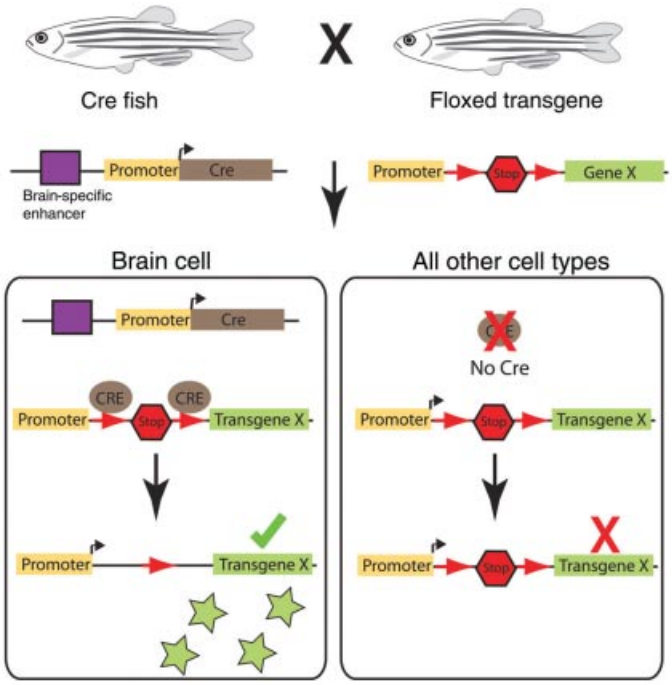


Figure 15.5. Activation of transgene expression by the Cre recombinase. Transgenic fish with cell-specific Cre expression are crossed to fish transgenic for a floxed Transgene X construct. Presence of a transcriptional stop or polyadenylation sequence (hexagon) between the floxed sequences prevents transcription of Transgene X. In transgenic fish containing both transgenes, CRE will recognize the loxP sequences (arrows) and excise the transcriptional stop sequence in a cell-specific manner. This allows for transcription to be initiated in Cre-expressing cells but not in others.

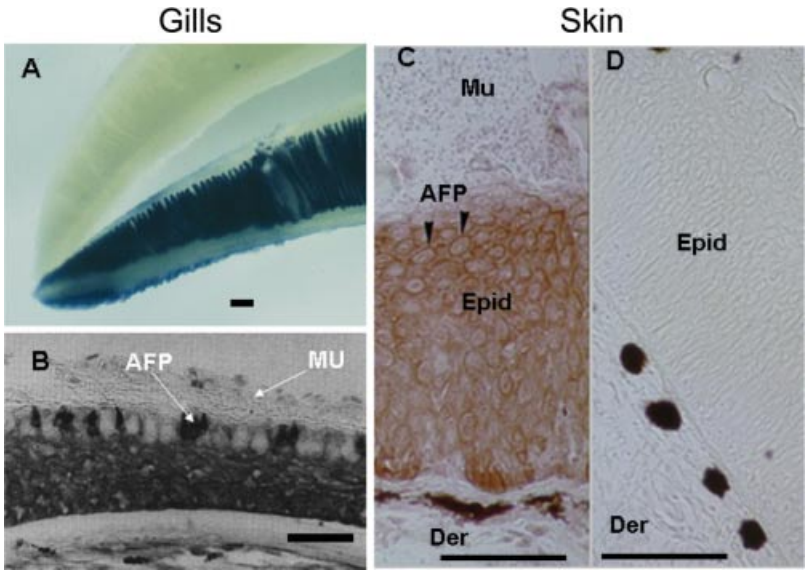


Figure 16.5. Tissue and cellular localization of AFP production in gill and skin epithelia of Newfoundland winter flounder. (**A**) Whole-mount in situ hybridization showing distribution of skin type AFP mRNA expression (dark stain) on gill filament lamellae. The lightly stained lamellae served as the control (Murray et al. 2002). (**B**) High magnification of a gill filament showing the cellular distribution of AFP-producing cells. (**C**) Extracellular localization of skin type AFP in the epidermis of winter flounder. (**D**) Control skin. All scale bars 100 μm. AFP in gill and skin samples was detected using antisera to skin type AFP. Normal rabbit serum was used as a control (Murray et al. 2002, 2003).

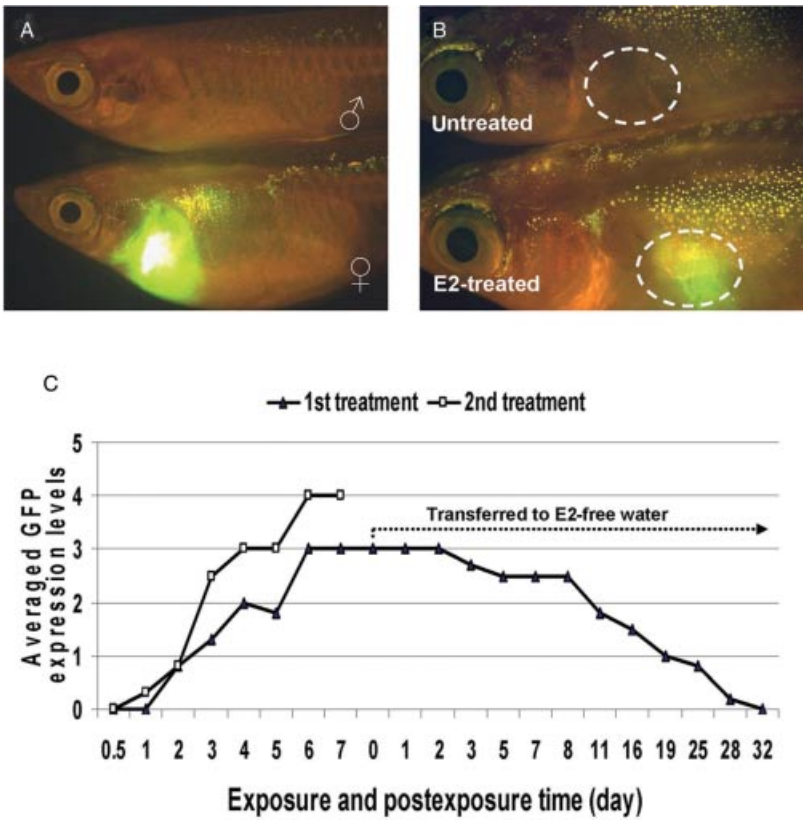


Figure 17.1. *Tg(mvtg1:gfp)* transgenic medaka. (**A**) Live adult male (top) and female (bottom) transgenic fish. Note that GFP expression is only in the liver of female transgenic fish. (**B**) Induction of GFP expression by exogenous 17β-estradiol (E2). Transgenic male fish were immersion-treated with 1 μg/L E2 for more than 12 hours and GFP expression was observed in the liver (bottom), while no GFP expression was observed in the liver area (circled) in untreated transgenic male fish (top). (**C**) Time course of GFP induction in transgenic male fish. Ten transgenic male fish were treated with 5 μg/L E2 for 7 days and GFP expression was induced to a relatively high level by 1 week. Thereafter, all of the fish were transferred into E2-free water on day 8 (or day 0 for E2-free water). GFP expression gradually decreased and was completely invisible by day 32 (see the 1st treatment curve with closed triangles). These fish were kept in estrogen-free water for another week and then treated with a second dose of E2 at the same concentration and GFP expression was reinduced (see the 2nd treatment curve with open squares). The details of the experiments and the estimation of relative levels of GFP expression have been described in our original publication (Zeng et al. 2005). Pictures and data are modified from Zeng et al. (2005).

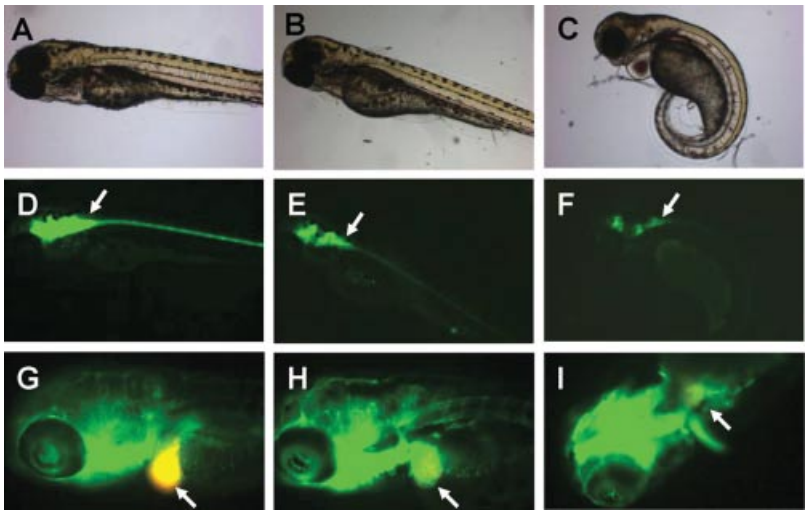


Figure 17.2. Use of fluorescent transgenic embryos/larvae for toxicological screen. (**A–F**) Estrogen-induced developmental toxicity in *Tg(nkx2.2a:mEGFP)* embryos. Transgenic embryos were treated with E2 at concentrations of 0 μg/L (ethanol vehicle) (**A**, **D**), 1.9 μg/L (**B**, **E**), and 2.7 μg/L (**C**, **F**) from 3 to 96 hpf (hour postfertilization). Panels **A**–**C** were captured under normal light while panels **E**–**H** were the same embryos/larvae captured under excitation light for GFP observations. Panels **D**–**F** show decreasing fluorescence and morphological disruption in the brain and spinal cord by increasing concentrations of E2. Arrows indicate the junction of brain and spinal cord. (**G**–**I**) Effect of E2 on vascularization and liver development in embryos from two-color double transgenic strain, *Tg(Fli:gfp; lfabp:rfp)*. Transgenic embryos were treated with E2 at concentrations of 0 μg/L (ethanol vehicle) (**G**), 1.4 μg/L (**H**), and 2.7 μg/L (**I**) from 3 to 96 hpf. Pictures were taken under conditions appropriate for GFP observation in the blood vessels and thus RFP expression in the liver is displayed in yellow (panel **G**) (arrows) under this condition. Decreasing fluorescence was observed in the liver with increasing concentration of E2, indicating affected vascularization and liver size.

Figure 19.6. GFP protein synthesis in the muscle tissue of a GFP transgenic fish (bottom). No GFP was detected in the muscle tissue of control fish (top).

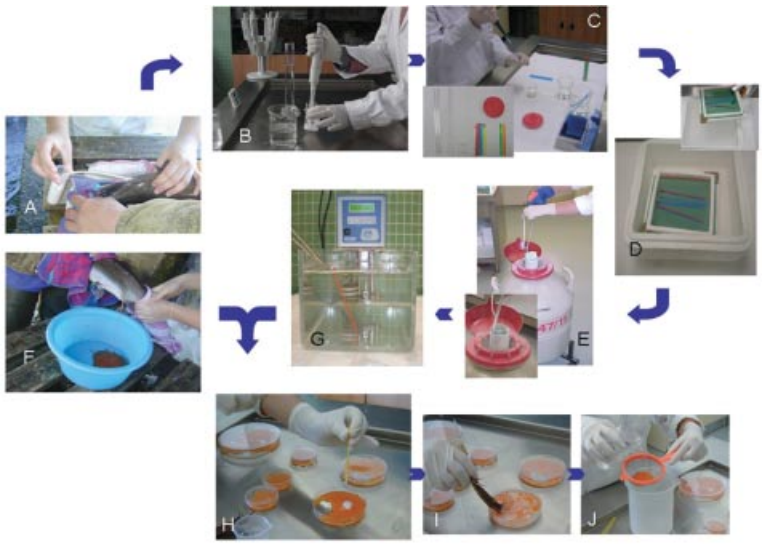


Figure 20.1. Sperm freezing process: (**A**) trout sperm extraction by canulation, (**B**) dilution in a cryoprotectant extender, (**C**) loading in 0.5 cc French straws (insert with different straws, cryovials, and PVA powder for straw sealing), (**D**) freezing over a floating device in a styrofoam box containing N_2l, (**E**) storage in a N_2l tank, (**F**) female stripping, (**G**) sperm thawing in a water bath, and (**H**–**J**) fertilization.

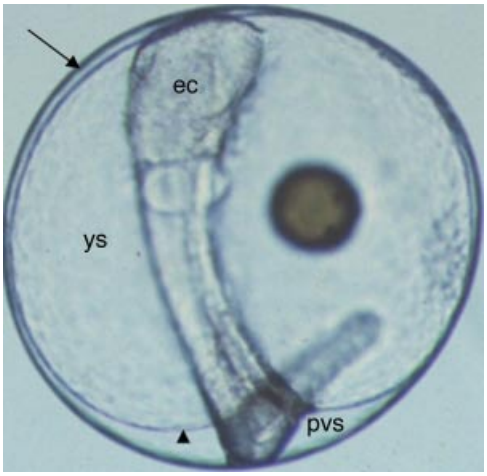


Figure 20.2. Turbot embryo at the tail bud stage showing the different envelopes and compartments: chorion (arrow), yolk syncytial layer (arrowhead), yolk sac (ys), perivitelline space (pvs), and embryo compartment (ec).

Figure 21.1. Atlantic salmon sea cage operation in Fortune Bay, Newfoundland, Canada.

Part 1
Broodstock Improvement

Chapter 1

Genomic Tools for Understanding the Molecular Basis of Production-Relevant Traits in Finfish

Marije Booman and Matthew L. Rise

OVERVIEW

Significant genomic resources (e.g., expressed sequence tag (EST) databases, DNA microarrays, single nucleotide polymorphism (SNP) genotyping platforms, bacterial artificial chromosome (BAC) libraries and BAC end sequences, genetic linkage maps, and physical maps) have been generated for several finfish species of importance to global aquaculture. Over the last few years, numerous articles (e.g., Cerdá et al. 2008; Koop et al. 2008), reviews (e.g., Douglas et al. 2006; Canario et al. 2008; Goetz and MacKenzie et al. 2008; Martin et al. 2008), and book chapters (e.g., Palti 2009; Rise et al. 2009; and several chapters in book *Aquaculture Genome Technologies*, 2007, edited by Z. Liu) have been published on the creation and application of finfish genomics resources. With the advent of next-generation sequencing (NGS) technologies, it is anticipated that finfish genomic resources will continue to expand.

For species of key importance to global aquaculture and fisheries (e.g., Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), channel catfish (*Ictalurus punctatus*), and common carp (*Cyprinus carpio*)), complete "genomics toolboxes" will be needed in order to take full advantage of the power of genomics in aquaculture research (e.g., for marker-assisted selection (MAS) of superior broodstock, development of optimal and sustainable feed formulations, development of maximally effective vaccines and therapeutants, etc.). Complete, high-quality reference genome sequences are critically important components of these toolboxes, and whole-genome sequencing projects are already underway for Atlantic salmon (Davidson et al. 2010) and catfish (Lu et al. 2011). As aquaculture finfish species' genomes are sequenced and assembled, and as we move into a postgenomics era for these species, bioinformatics will undoubtedly play an ever-increasing role in the success of aquaculture genomics research.

Because of the aforementioned publications on aquaculture finfish genomic resources, we do not attempt to provide an exhaustive summary of the field. Rather, the aim of this chapter is to cover new territory. Since we have been involved in the Atlantic Cod Genomics and Broodstock Development Project (CGP, http://codgene.ca), we use CGP

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

examples to illustrate different aspects of finfish functional genomics tools. In Section "Targeted, Trait-Relevant Gene Discovery," we discuss CGP examples of how suppression subtractive hybridization (SSH) cDNA libraries were employed for discovery of Atlantic cod genes that were involved in defense responses. In Section "The Application of Microarray Technology in Finfish Aquaculture and Research," we discuss microarray technology, including brief overviews of current finfish microarray platforms, the construction of the CGP Atlantic cod oligonucleotide microarray focusing on trait-relevant transcripts, and software for the analysis of microarray data. We discuss how these functional genomics tools will likely play key roles in genomics toolboxes by allowing the identification of the best targets for MAS of superior aquaculture broodstock. We end the chapter with recommendations and future directions for finfish microarray (and other genomics) research.

TARGETED, TRAIT-RELEVANT GENE DISCOVERY

Normalized (e.g., Rise et al. 2004b; Gahr et al. 2007), unnormalized (e.g., Goetz et al. 2009), and SSH (e.g., Rise et al. 2008, 2010; Feng et al. 2009; Hori et al. 2010) cDNA libraries have been utilized in large-scale gene discovery efforts for several aquaculture finfish and shellfish species (see Chapters 2 (by Robalino et al.), 7 (by Douglas), and 8 (by Dixon and Becker) of this book for information on targeted gene discovery in aquaculture species). The numbers of ESTs currently in GenBank for selected aquaculture fish species are shown in Table 1.1.

For each aquaculture-relevant finfish species, ESTs are generally contributed from several researchers or genomics projects (e.g., see Rise et al. 2007 for information on origins of salmonid EST resources). Each of the large-scale aquaculture finfish genomics research projects to date has employed a particular strategy for cDNA library

Table 1.1. Expressed Sequence Tag (EST) Collections of Selected Aquaculture Fish Species.

Selected Orders of Teleosts	Examples of Aquaculture Species With Genomic Resources	Number of ESTs in GenBank*
Superorder Acanthopterygii		
Perciformes	Nile tilapia (*Oreochromis niloticus*)	117,222
	Gilthead seabream (*Sparus aurata*)	67,670
	European seabass (*Dicentrarchus labrax*)	55,835
	Yellow perch (*Perca flavescens*)	21,968
Superorder Ostariophysi		
Siluriformes	Channel catfish (*Ictalurus punctatus*)	354,466
	Blue catfish (*Ictalurus furcatus*)	139,475
Cypriniformes	Common carp (*Cyprinus carpio*)	34,067
Superorder Paracanthopterygii		
Gadiformes	Atlantic cod (*Gadus morhua*)	229,090
Superorder Protacanthopterygii		
Salmoniformes	Atlantic salmon (*Salmo salar*)	498,212
	Rainbow trout (*Oncorhynchus mykiss*)	287,967
	Chinook salmon (*Oncorhynchus tshawytscha*)	14,173
	Sockeye salmon (*Oncorhynchus nerka*)	11,389
	Coho salmon (*Oncorhynchus kisutch*)	4,942

*Numbers of ESTs were acquired from NCBI Entrez Taxonomy on November 4, 2010.

construction and gene discovery. For example, the CGP generated over 138,000 ESTs from a total of 23 normalized libraries and over 20,000 ESTs from a total of 19 SSH libraries (Bowman et al. 2011). Of the ∼158,000 ESTs generated by the CGP, approximately 45,000 were from cDNA libraries that were designed for the discovery of cod transcripts that responded to pathogens or pathogen-associated molecular patterns (PAMPs) (Bowman et al. 2011); of these, approximately 10,000 ESTs came from cod SSH libraries that were enriched for transcripts that respond to nodavirus and/or polyriboinosinic polyribocytidylic acid (pIC, a synthetic double-stranded RNA that induces interferon pathway genes; Rise et al. 2008, 2010; Bowman et al. 2011), and over 4300 ESTs came from cod SSH libraries enriched for transcripts that respond to bacterial antigens (Feng et al. 2009; Fig. 1.1). Many of the transcripts

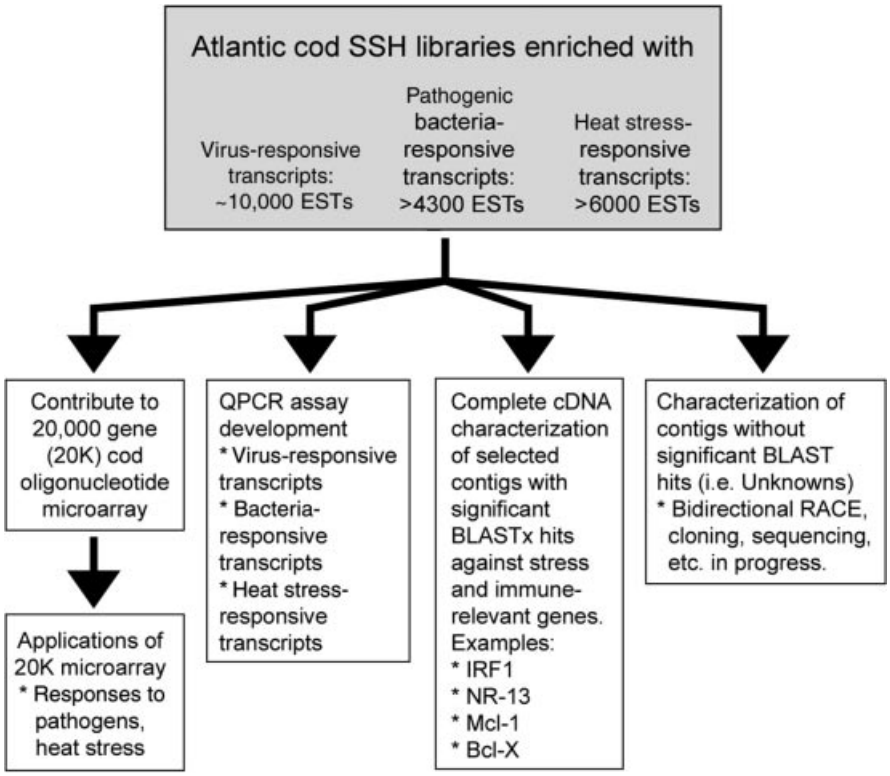


Figure 1.1. Functional genomics workflow in the Atlantic Cod Genomics and Broodstock Development Project (CGP). Suppression subtractive hybridization (SSH) libraries were designed for the targeted discovery of cod transcripts that respond to virus (Rise et al. 2008, 2010), bacterial antigens (Feng et al. 2009), or heat stress (Bowman et al. 2011; Hori et al. 2010). Many trait-relevant genes identified in SSH libraries were included on the CGP 20K oligonucleotide microarray (Booman et al. 2011), making this a valuable new resource for defense-relevant functional genomics research on Atlantic cod. Quantitative reverse transcription – polymerase chain reaction (QPCR) assays were developed and used to study transcript expression of many SSH-identified cod genes (e.g., Feng et al. 2009; Rise et al. 2008, 2010; Booman et al. 2011; Hori et al. 2010). In addition, several SSH-identified defense-relevant transcripts (e.g., interferon regulatory factor 1 (IRF1) and anti-apoptotic Bcl-2 family genes NR-13, Mcl-1, and Bcl-X1) were further characterized using bidirectional rapid amplification of cDNA ends (RACE) and other molecular techniques. (From Feng et al. (2009) and Feng and Rise (2010).)

identified in the CGP's cDNA libraries designed for targeted discovery of immune-relevant genes were included on the CGP 20,000 gene (20K) oligonucleotide microarray (Booman et al. 2011; see section of this chapter entitled "Design of a New Microarray for Aquaculture Research: The Atlantic Cod 20K Oligonucleotide Microarray"), making this microarray platform a valuable new tool for functional genomics research on Atlantic cod defense responses.

An obvious goal of any large-scale, gene discovery project is to identify all of the genes in the species of interest. Although the EST collections of some aquaculture finfish species are quite large (e.g., >200,000 ESTs for Atlantic salmon, channel catfish, rainbow trout, and Atlantic cod; Table 1.1), it is likely that they are still missing important, trait-relevant transcripts. For example, if high-quality cDNA libraries enriched for gill transcripts that are responsive to infection by a fungal pathogen are not incorporated into a finfish species' genomics research plan, then that species' EST collection may be missing defense-relevant transcripts that respond specifically to fungi. The same may be said of species for which cDNA libraries enriched with transcripts that respond to toxicants (e.g., dioxins, heavy metals, pesticides) or other stressors (e.g., heat stress, cold stress, hypoxia, etc.) have not been built and characterized. Presumably, with adequate bioinformatics support, complete genome sequencing, reference genome assembly, and next-generation-based deep sequencing of transcripts will facilitate the complete characterization of finfish transcriptomes. However, until these resources are available, targeted gene discovery tools (e.g., SSH libraries) can be extremely effective means of ensuring that key genes involved in production-relevant traits of interest (e.g., enhanced growth rate, resistance to heat stress, and resistance to pathogens) are represented in aquaculture species' EST collections and the genomics resources arising from those EST sets (e.g., DNA microarrays and SNP genotyping platforms).

THE APPLICATION OF MICROARRAY TECHNOLOGY IN FINFISH AQUACULTURE AND RESEARCH

The establishment of large EST collections (e.g., Table 1.1) has facilitated the development of finfish DNA microarrays that have become important tools in finfish aquaculture genomics research. In this section, we give an overview of the microarray platforms that are currently described in the literature. We briefly discuss the development of the CGP 20K Atlantic cod oligonucleotide microarray as an example of a platform focusing on trait-relevant transcripts for aquaculture research. Next, we discuss tools for microarray data analysis. Finally, we end this section with future perspectives and some recommendations for future microarray research.

Overview of Microarray Platforms and Their Use in Aquaculture

A search through NCBI's PubMed and Gene Expression Omnibus (GEO) databases shows that there are over 40 finfish species that have been studied using microarrays and that research areas vary from biomedicine and ecotoxicology to nutrigenomics and immunology. The largest numbers of microarray platforms are available for zebrafish and salmonids. Some microarray platforms are most useful in specific research areas as they are limited to transcripts from specific tissues or developmental stages, or contain only transcripts involved in specific functions or transcripts responsive to a specific treatment or condition. Other microarray platforms represent the transcriptome of multiple tissues, developmental stages, treatments, and conditions and are useful for a wider range of research areas. These platforms are often made available either through companies like Agilent, Affymetrix, and NimbleGen or through research institutes, as with the consortium for Genomics Research on All Salmonids (cGRASP) arrays (Rise et al. 2004b; von Schalburg et al. 2005; Koop et al. 2008) and the CGP 20K Atlantic cod oligonucleotide microarray (Booman et al. 2011). Table 1.2 gives an overview of selected finfish species and microarray platforms on the basis of their economic importance and their usefulness in aquaculture research.

Microarrays have successfully been used to study multiple aspects of finfish biology that are of interest to aquaculture. Examples are the response to stress such as hypoxia (e.g., van der Meer et al. 2005), temperature changes (e.g., Vornanen et al. 2005; Chou et al. 2008), or handling (e.g., Krasnov et al. 2005; Wiseman et al. 2007); the immune response to different types of pathogens such as bacteria (e.g., Rise et al. 2004a; Gerwick et al. 2007), viruses (e.g.,

Table 1.2. Overview of Selected Finfish Microarray Platforms.

Order	Species	Technology	Microarray Platform
Cypriniformes	*Cyprinus carpio* (common carp)	Spotted cDNA	CarpARRAY v5 26K (Williams et al. 2008)
	Danio rerio (zebrafish)	Spotted oligo	Compugen/Sigma-Genosys 16K (e.g., Malek et al. 2004; Rawls et al. 2004); MWG 14K (e.g., van der Meer et al. 2005; Chou et al. 2008); Compugen/Sigma-Genosys/MWG/Operon 35K (e.g., Pei et al. 2007)
		In situ oligo	Leiden custom Agilent 44K (Stockhammer et al. 2009; GPL7735); Agilent zebrafish 21K (GPL7244); Agilent zebrafish 44K (GPL3701); Affymetrix zebrafish 15K (GPL1319); Nimblegen zebrafish 32K (GPL5746)
Gadiformes	*Gadus morhua* (Atlantic cod)	Spotted cDNA	CodStress 0.7K (Lie et al. 2009); IMR Atlantic cod 16K (Edvardsen et al. 2011)
		Spotted oligo	CGP Atlantic cod 20K v1.0 (Booman et al. 2011; GPL 10532)
Perciformes	*Dicentrarchus labrax* (European sea bass)	Spotted cDNA	INRA-Agenae rainbow trout 9K (Govoroun et al. 2006; Darias et al. 2008; GPL3650)
	Sparus aurata (gilthead seabream)	Spotted cDNA	Embryonic/larval cDNA 10K (Sarropoulou et al. 2005; GPL1516)
		In situ oligo	Padova custom Agilent 40K (Ferraresso et al. 2008; GPL6467)
Pleuronectiformes	*Hippoglossus hippoglossus* (Atlantic halibut)	Spotted oligo	Pleurogene halibut 10K (Douglas et al. 2008; GPL6361)
	Paralichthys olivaceus (Japanese flounder)	Spotted cDNA	Immune-related 0.9K (Kurobe et al. 2005); immune-related v2 1.1K (Matsuyama et al. 2007); custom digital genomics 0.3K (Nakayama et al. 2008)
	Psetta maxima (turbot)	Spotted cDNA	Virus-stimulated 2K (Park et al. 2009)
	Solea senegalensis (Senegalese sole)	Spotted oligo	Pleurogene Sole 5K (Cerdá et al. 2008)
Salmoniformes	*Oncorhynchus mykiss* (rainbow trout)	Spotted cDNA	Stress-responsive SFA salmonid V1 1.3K (Krasnov et al. 2005; GPL1212)*; Immunochip SFA salmonid V2 1.8K (Jørgensen et al. 2008; GPL6154)*; cGRASP salmonid 3.6K (Rise et al. 2004b; GPL966)*; cGRASP salmonid v2 16K (von Schalburg et al. 2005; GPL2716)*; cGRASP salmonid 32K (Koop et al. 2008; GPL8904)*; INRA-Agenae rainbow trout 9K (Govoroun et al. 2006; GPL3650); stress-responsive UWVJLAB RT 150 0.1K (Wiseman et al. 2007; GPL3713)

(*Continued*)

Table 1.2. *Continued*

Order	Species	Technology	Microarray Platform
		Spotted oligo	Stress-responsive OSUrbt v2 1.6K (Tilton et al. 2005; GPL5478); Custom rainbow trout 21.5K (Olohan et al. 2008)
		In situ oligo	Custom Agilent rainbow trout 37K (Salem et al. 2008; GPL8205); Gothenburg rainbow trout 15.5K (Gunnarsson et al. 2009; GPL8254)
	Oncorhynchus tshawytscha (Chinook salmon)	Spotted cDNA	cGRASP salmonid v2 16K (von Schalburg et al. 2005; GPL2716)*
	Salmo salar (Atlantic salmon)	Spotted cDNA	Stress-responsive SFA salmonid V1 1.3K (Krasnov et al. 2005; GPL1212)*; Immunochip SFA salmonid V2 1.8K (Jørgensen et al. 2008; GPL6154)*; cGRASP salmonid 3.6K (Rise et al. 2004b; GPL966)*; cGRASP salmonid v2 16K (von Schalburg et al. 2005; GPL2716)*; cGRASP salmonid 32K (Koop et al. 2008; GPL8904)*; IMB Atlantic salmon 4K (Ewart et al. 2005; GPL2844); TRAITS/SGP Atlantic Salmon 17K (Martin et al. 2007); lipid metabolism 73 genes (Jordal et al. 2005)
		In situ oligo	Agilent Atlantic salmon 44K (GPL7303)
Siluriformes	*Ictalurus furcatus* (blue catfish)	In situ oligo	Custom Nimblegen catfish 28K (Peatman et al. 2007; GPL4476)†
	Ictalurus punctatus (channel catfish)	Spotted cDNA	Channel catfish brain 0.6K (Ju et al. 2002); UMSMED channel catfish 9.2K (Majji et al. 2009; GPL6997)
		In situ oligo	Custom Nimblegen channel catfish 19K (Li and Waldbieser 2006; GPL2814); Custom Nimblegen Catfish 28K (Peatman et al. 2007; GPL4476)†

Source: NCBI's GEO database and PubMed database.
Note: This table lists the microarray platforms that are of interest to aquaculture research. Microarray platforms are grouped by order, species, and technology (spotted cDNA, spotted oligonucleotide, or in situ synthesized oligonucleotide). References include the publication describing the microarray design and, where available, the GEO platform accession number (in "GPLxxxx" format).
*Combined *S. salar* and *O. mykiss*.
†Combined *I. furcatus* and *I. punctatus*.

MacKenzie et al. 2008; Workenhe et al. 2009), or parasites (e.g., Morrison et al. 2006; Skugor et al. 2008; Young et al. 2008); the influence of nutrition (e.g., Leaver et al. 2008; Murray et al. 2010); and gene expression profiles related to egg quality (e.g., Bonnet et al. 2007) and growth (e.g., Rise et al. 2006; Gahr et al. 2008; Devlin et al. 2009). As previously mentioned, there have been several reviews and chapters on the applications of finfish DNA microarrays (e.g., Douglas 2006; Rise et al. 2007, 2009; Goetz and MacKenzie et al. 2008). Other chapters in this book review literature pertaining to the use of finfish microarrays to study fish egg and embryonic gene expression (Chapter 11, Traverso et al.) and fish immune responses (Chapter 6, Johnson and Brown). We refer the reader to these sources for reviews of the microarray-related aquaculture literature.

Design of a New Microarray for Aquaculture Research: The Atlantic Cod 20K Oligonucleotide Microarray

For most aquaculture-relevant finfish species, a whole-genome sequence is unavailable, and sequences for microarray construction are provided by EST collections. Therefore, the possibilities for application of a newly developed microarray platform in aquaculture research largely depend on the characteristics of the underlying EST collection and the process of selection of sequences from that collection.

An example of a microarray platform that was specifically designed for aquaculture research is the CGP Atlantic cod 20K oligonucleotide microarray platform (Booman et al. 2011). It is based on the CGP EST collection, discussed earlier in this chapter, which contains over 150,000 ESTs (Bowman et al. 2011). This EST collection represents 42 cDNA libraries from 12 tissue types and 4 developmental stages; some of the libraries were constructed with tissues sampled from fish that were exposed to stressors or to bacterial or viral immunostimulants. This diversity, together with its size, made the CGP EST collection very well suited for the construction of a microarray that contains a wide range of trait-relevant transcripts for aquaculture research. To prevent redundancy on the microarray and maintain the sequence diversity, the ESTs were first clustered and assembled into unique transcripts, which were then used to design oligonucleotide probes. For the selection of the final set of 20,000 probes, sequences were given priority if they had an informative annotation, if they were represented by a large number of ESTs, or if they were unannotated but represented by a large number of ESTs from the SSH libraries enriched for stress- or immune-responsive transcripts. This selection process ensured not only that the microarray contains enough annotated genes to enable functional interpretation of results but also that it contains unannotated, and therefore possibly novel, genes that have a possible role in traits relevant to aquaculture, such as resistance to stress and pathogens. Within the CGP, this microarray is currently being used in a number of different projects to identify transcripts associated with egg quality, response to heat stress, and response to viral and bacterial immunostimulation.

Microarray Data Analysis Tools

Most data acquisition programs such as Imagene (BioDiscovery) or GenePix (MDS Analytical Technologies) offer basic functionality for data normalization and visualization. For a more in-depth analysis of the acquired microarray data, specialized commercial software packages are available (e.g., Agilent's GeneSpring GX), which provide full data analysis functionality, including data normalization, identification of differentially expressed genes, and pathway analysis. Most commercial software is relatively easy to use with a good graphical user interface, but it is also expensive and often after the release of a new version, the older versions are no longer supported. Part of the functionality of these packages, such as functional analysis or pathway analysis, relies on the annotation options provided by the package, which are usually limited to model species.

There are noncommercial, free alternatives for most commercial programs (summarized in Table 1.3), such as Significance Analysis of Microarrays (SAM) (Tusher et al. 2001) for class comparison and Gene Set Analysis, and Genesis (Sturn et al. 2002) for clustering. A large source of noncommercial microarray analysis tools is the Bioconductor project, an open source software project "to provide tools for the analysis and comprehension of genomic data" (Gentleman et al. 2004). These tools

Table 1.3. Microarray Data Analysis Noncommercial Software.

Program	Reference	URL
Data visualization and processing		
marray	Yang and Paquet 2005	www.bioconductor.org
limma	Smyth 2005	www.bioconductor.org
Statistical analysis of gene expression differences		
limma	Smyth 2005	www.bioconductor.org
Siggenes	Schwender et al. 2006	www.bioconductor.org
multtest	Pollard et al. 2005	www.bioconductor.org
Significance analysis of microarrays	Tusher et al. 2001	www-stat.stanford.edu/∼tibs/SAM/
Clustering		
Cluster/TreeView	Eisen et al. 1998	http://rana.lbl.gov/eisen/?page_id=42
Cluster 3.0	de Hoon et al. 2004	http://bonsai.ims.u-tokyo.ac.jp/∼mdehoon/software/cluster/
Java TreeView	Saldanha 2004	http://jtreeview.sourceforge.net/
Genesis	Sturn et al. 2002	genome.tugraz.at/genesisclient/genesisclient _description.shtml
Functional analysis of gene lists		
Gene set analysis	Efron and Tibshirani 2007	www-stat.stanford.edu/∼tibs/GSA/
Gene set enrichment analysis	Subramanian et al. 2005	www.broadinstitute.org/gsea/
DAVID	Huang et al. 2009	david.abcc.ncifcrf.gov/
GenMAPP	Salomonis et al. 2007	www.genmapp.org/
Blast2GO	Götz et al. 2008	www.blast2go.org

are based on the statistical computing language R (R Development Core Team 2010). Since most do not have a graphical user interface, they have a steeper learning curve than most commercial software, but there is very strong community-based support. Also, Bioconductor and R tools are customizable, so they could be adapted to work with custom array formats and nonmodel species. Following is a short description of the programs listed in Table 1.3 by category.

Data Visualization and Processing

The Bioconductor packages marray (Yang and Paquet 2005) and limma (Smyth 2005) both contain functions for data visualization and normalization. Both packages share the same origins and so there is some overlap, but their functions for data visualization and normalization are mainly complementary. Limma has an optional graphical user interface (limmaGUI; Wettenhall and Smyth 2004).

A typical first step in quality control of array data would be to plot several measurements as a color range onto a spatial image (such as signal or background intensity, spot size or shape, or quality flags; the marray package has more options for spatial image plotting than limma). In a spatial image, the layout of the array is shown as a collection of squares, each representing a single spot location. Plotting, for instance, the background signal intensities onto this spatial image gives a quick overview of spatial artifacts present on the array. There are also functions for creating box plots and scatter plots, such as the typical M–A plots, with optional smoothed fits.

Both packages have several options for data normalization. The results from the various plots will help determine what type of normalization is necessary (e.g., a global normalization or a normalization per print-tip).

The limma package has additional functionality for different types of background correction and the

assignment of quality weights to spot measurements. It also has gene expression analysis functionality (discussed in the next section).

Statistical Analysis of Gene Expression Differences

When determining which genes are differentially expressed between two groups in a microarray experiment, performing a simple *t*-test on each gene is not allowed. Because a high number of genes are tested at the same time, more sophisticated statistics are needed, e.g., to correct for multiple testing. Statistical analysis can also be complicated by the experimental design, such as when analyzing a time series. Several software packages are available for microarray analysis. Some provide only basic functionality and require considerable statistical knowledge from the researcher, while others are very user-friendly and guide the researcher through the process.

The Bioconductor package multtest (Pollard et al. 2005) is an example of a basic statistical package. When comparing gene expression levels between two groups, this package can be used to run parametric or nonparametric tests, and it provides several methods for applying multiple testing correction to the results. However, the package does not give much guidance as to which test statistic and which correction procedure are best suited for the specific dataset, and there is a large number of parameters that must be set correctly for each test. Therefore, help from a statistician is recommended when using this package.

The limma package (Smyth 2005) can be used to determine differentially expressed genes in a variety of experimental setups such as one-color arrays, universal reference designs, and direct comparisons. It uses linear model fitting, empirical Bayes smoothing, and multiple testing correction. It also includes methods to deal with arrays that contain duplicate spots. As mentioned, it includes an optional graphical user interface for more convenient analysis.

One of the most widely used software packages for gene expression analysis is SAM (Tusher et al. 2001). It is supplied as an add-in for Microsoft Excel, which makes it very easy to use, even for inexperienced researchers. It can correlate gene expression differences to a variety of response variables, including two-class comparisons (e.g., control versus treatment), multiclass comparisons (e.g., different types of treatment), quantitative variables (e.g., weight), and time-course experiments. It can also handle one-class experiments, which are used in case of a direct comparison experiment (e.g., where control and treatment samples are hybridized together on the same array). The user has control over which test statistic is used (parametric or nonparametric). SAM uses repeated permutations of the response variables to determine *q*-values (which are representations of *p*-values that are corrected for multiple testing) and false discovery rates (FDRs), and the list of genes that are significantly associated with the response variable is determined by a user-defined cutoff value for the FDR. The effect of changing the FDR cutoff on the list of significant genes is visualized by a SAM plot, which allows the user to make the best decision to acquire desired results. Apart from the control over the FDR, the user can also add an optional fold change requirement for the list of significant genes. When very large datasets must be analyzed, the Excel add-in might become very slow or even unresponsive. For analysis of these large datasets, the Bioconductor package siggenes (Schwender et al. 2006) is available, which enables users to run the SAM algorithm in R.

Clustering

Clustering of microarray data is used to identify groups of samples or genes with related gene expression patterns. Often, the whole dataset is used in a process called unsupervised hierarchical clustering, where there is no information provided to the clustering algorithm regarding specific sample classes or gene classes. Using unsupervised hierarchical clustering, one can gain information on whether there are specific subgroups within the total sample set, and on which genes show similar expression patterns over all samples. Clustering of genes is often used as a starting point for finding genes that are involved in specific pathways or share similar functions. Clustering can also be performed using a list of differentially expressed genes. This can provide more information on, e.g., the heterogeneity of sample subgroups. The results of hierarchical clustering are often displayed as a heat map, where the gene expression values are represented by colors.

Apart from hierarchical clustering, there are a number of other clustering algorithms such as *k*-means clustering (where the user specifies a desired

number of clusters), self-organizing maps (SOM), and principal component analysis (PCA). In order to cluster the data, the user has to choose a distance or similarity measurement (e.g., Pearson correlation or Euclidian distance) and a clustering algorithm.

The most well-known clustering programs for microarray analysis are Michael Eisen's Cluster (for clustering) and TreeView (for visualization of the clustering results) (Eisen et al. 1998); the original programs have been continued as open-source versions Cluster 3.0 (de Hoon et al. 2004) and Java TreeView (Saldanha 2004). These programs can perform hierarchical, SOM, *k*-means, and PCA clustering using a number of parametric and nonparametric distance measurements. The output of the Cluster program is numerical and the output files can be loaded into TreeView to visualize the results in a heat map.

A second program that is recommended for clustering is Genesis (Sturn et al. 2002). This is a Java-based program that can perform a large number of different clustering algorithms using a variety of distance or similarity measurements. Advantages of Genesis are detailed control over the visualization of the results and convenient options for marking samples or genes and for selecting a specific cluster for further analysis, e.g., to turn it into a separate gene list or to investigate behavior of genes in this cluster in other clustering algorithms.

Functional Annotation of Gene Lists

The final step in microarray data analysis is to make biological sense of the acquired data. When a significant gene list has been determined using gene expression analysis, as described in Section "Statistical Analysis of Gene Expression Differences," it is often not easy to discover the biological meaning behind the selected genes.

There are programs available that can annotate a list of differentially expressed genes and determine if certain annotations are overrepresented by comparing the proportion of genes with a specific annotation in the list of significant genes to the proportion of genes with the same annotation in the background gene list (often representing either the whole genome or the complete list of genes present on the array platform). One such program is the Database for Annotation, Visualization and Integrated Discovery (DAVID; Dennis et al. 2003; Huang et al. 2009), which can be used online. It uses a modified Fisher-exact *p*-value to determine overrepresentation of annotations in the list of significant genes. It can also analyze overrepresentation of gene sets. Furthermore, it is a very useful program to easily annotate a list of genes with information from a large number of different databases.

Another option is to perform a gene set enrichment analysis. This differs from the previously discussed overrepresentation analysis because it does not require that a list of significant genes be determined first; instead, it uses the complete dataset. In a gene set enrichment analysis, instead of identifying single genes that are differentially expressed between two groups, the genes are first grouped into gene sets that can be provided by the user (e.g., based on biological functions, pathways, or transcription factor-binding sites) and then the analysis determines which of these gene sets show significant concordant differences between two sample groups. The advantage is that, although multiple genes from one pathway may not reach statistical significance on their own, they might be significant when regarded as a group. Examples of programs that can perform gene set enrichment analysis are GSA (Efron and Tibshirani 2007), which is available as a module in SAM (Tusher et al. 2001), and GSEA (Mootha et al. 2003; Subramanian et al. 2005), which is available as a Java-based program (Subramanian et al. 2007) or as an R package.

A third group of functional annotation programs are the pathway analysis programs. These programs use databases of known pathways, such as Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) and Biocarta (www.biocarta.com), and project the gene expression data onto these pathways to give a visualization of the regulation of all the genes within one pathway. A good example is GenMAPP (Doniger et al. 2003; Salomonis et al. 2007). This program can also be used to construct new pathways and to perform overrepresentation analysis.

There is one drawback to annotation-based analysis programs for aquaculture research. They all work with a certain "standard" annotation that they use to couple gene expression data to the gene sets and pathways. Most programs use Unigene or Entrez human gene symbols. Some have additional

databases for other model organisms such as mouse, and some also contain databases to convert, for instance, Affymetrix probeset IDs to their standard annotation. If these tools are to be used for aquaculture research, the researcher needs to be able to convert the ID used on the aquaculture microarray platform to one of these standard identifiers. In most cases, this will probably mean that the array must be annotated with the closest human putative orthologs. Therefore, these tools must be used with caution, as putative orthologs do not always share the same function.

For researchers working on nonmodel species, there is a program, Blast2GO, that can provide automated Gene Ontology (GO) annotation for a large set of sequences (Conesa et al. 2005; Götz et al. 2008). This program uses the NCBI Blast database to annotate the sequences and then uses the Blast results to map the sequences to GO terms and KEGG pathways. Functional analysis is provided by the program in the form of charts, graphs, and overrepresentation analysis. In addition, the annotations provided by Blast2GO can be used for further analysis by the tools discussed.

Recommendations and Future Directions

There are some recommendations to make for future microarray research. First of all, the research community would benefit greatly if the platforms that are developed would be made publicly available. This would reduce the redundancy of platforms that are produced from the same oligo sets, such as is currently seen with the zebrafish oligo arrays. Having a small number of "standard," widely applicable arrays per species would also make comparison of results between studies (metadata analysis) more feasible. Second, although we are aware that not all journals currently require it, all published microarray data should be entered into a database such as NCBI's GEO. The standardization of information that is required and provided by a database like GEO ensures that there is unambiguous and detailed information available about microarray experiments and that they are compliant to the Minimum Information About a Microarray Experiment (MIAME) standard (Brazma et al. 2001). In the case of platform entries, this would also benefit researchers searching for a suitable platform for their experiments.

The development of well-annotated whole transcriptome microarrays for all aquaculture finfish species would be very beneficial for aquaculture research. Currently, the finfish species with the most complete microarray platforms is the zebrafish, because this species is well characterized both on a genomic and transcriptomic level. (For example, NCBI's Taxonomy Browser shows 1,481,936 publicly available *Danio rerio* ESTs.) To reach the same goal for other finfish species, large EST databases must be established. To ensure that these databases cover the entire transcriptome, they should include ESTs from a variety of cDNA libraries (e.g., normalized tissue- and developmental stage-specific cDNA libraries; SSH libraries enriched for transcripts of interest, e.g., immune- and stress-responsive transcripts), subjected to deep sequencing using a NGS platform such as Roche/454's GS FLX Titanium, Illumina/Solexa's GA, or Life/APG's SOLiD 3 (reviewed by Metzker 2010). Combined with high-throughput NGS-based characterization of aquaculture finfish whole genomes, this should enable the development and annotation of whole transcriptome microarrays for species important in aquaculture research.

When high-quality reference genome sequences are available for aquaculture finfish species, high-throughput NGS-based transcriptome analysis (i.e., RNA-seq) will be utilized for some global transcript expression studies. However, DNA microarrays will likely continue to be utilized for global gene expression studies for finfish species lacking high-quality reference genome sequences. In addition, microarrays will likely continue to be utilized for complex experimental designs (e.g., multiple treatments, sampling time points, etc.), as they currently allow global transcript expression studies to be performed on large numbers of biological replicates at reasonable costs using established data analysis methods (e.g., Booman et al. 2011).

The development of functional genomics resources such as targeted gene discovery tools (e.g., SSH libraries) and gene expression microarrays, combined with complementary technologies (e.g., NGS and quantitative reverse transcription–polymerase chain reaction (QPCR)), are helping researchers to take a large step toward identifying genes that are involved in traits that are

important in aquaculture, such as resistance to disease and stress and good growth characteristics. For example, suites of trait-relevant genes (e.g., upregulated in pathogen-resistant Atlantic cod spleen compared to pathogen-susceptible Atlantic cod spleen) identified using functional genomics experiments can be validated and further studied at the transcript expression level in numerous individuals and families using high-throughput QPCR. In addition, trait-relevant candidate genes (exons, introns, and regulatory regions) can be subjected to NGS-based targeted resequencing with pooled templates from individuals in families enrolled in broodstock development programs to identify SNPs that may be used in MAS of finfish with superior production traits (Fig. 1.2). Aquaculture genomics projects can increase the likelihood of identifying useful, trait-correlated SNPs for MAS if high-throughput SNP

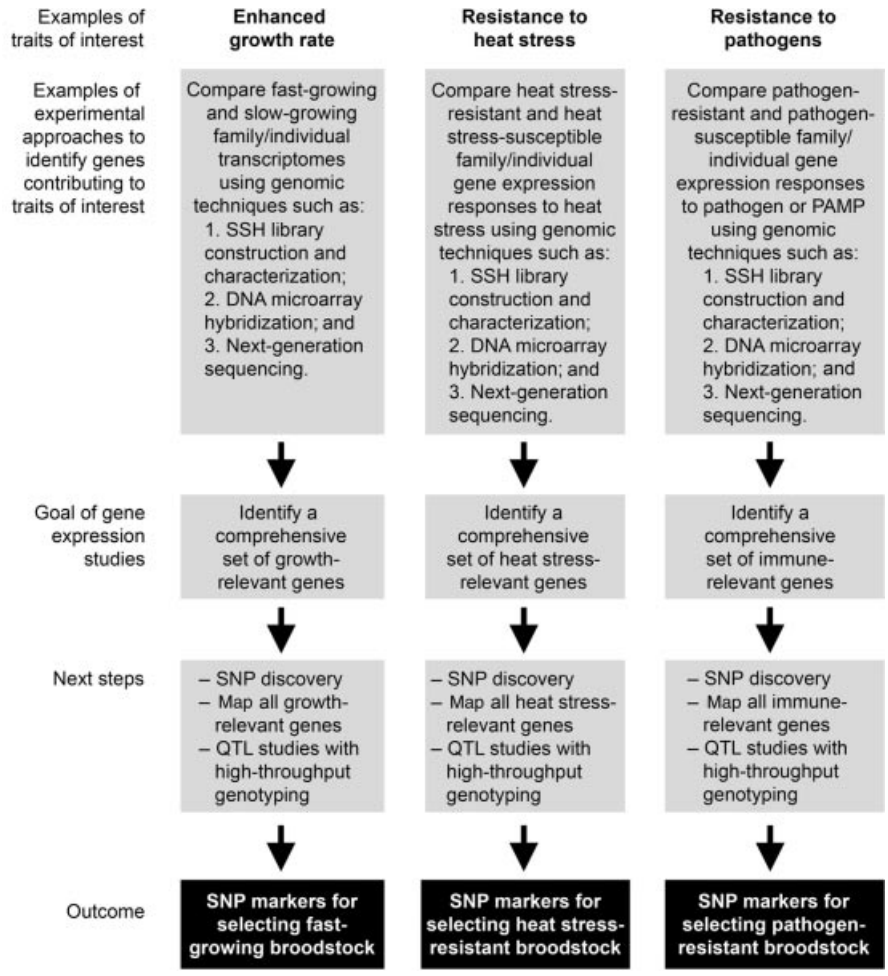


Figure 1.2. Examples of experimental approaches to (1) identify genes contributing to aquaculture-relevant traits (e.g., rapid growth, or resistance to pathogens or environmental stressors); and (2) identify single nucleotide polymorphisms (SNPs) associated with trait-relevant candidate genes (e.g., exonic, intronic, or in regulatory regions) that may be useful in marker-assisted selection (MAS) of finfish with superior production traits.

genotyping (e.g., using SNP platforms by companies such as Illumina) is employed to screen large numbers of candidate SNPs with large numbers of individuals.

In addition to previously mentioned applications, functional genomics studies (e.g., using gene expression microarrays) will also be helpful in the development of nonlethal diagnostic tests for pathogen infection and carrier state. To help diagnosis of pathogen infection and to increase our understanding of biological processes that take place during infection of finfish, some pathogen-specific microarrays have been developed (Chen et al. 2006; Nash et al. 2006).

Most current finfish microarray platforms are used for gene expression analysis. However, regulation takes place on many other levels, such as at the protein level or by epigenetic mechanisms. In the future, other array technologies such as antibody arrays or ChIP-on-chip arrays should be able to give a more complete view of gene and protein regulation associated with important traits.

REFERENCES

Bonnet E, Fostier A, and Bobe J. 2007. Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics. 8: 55.

Booman M, Borza T, Feng CY, Hori TS, Higgins B, Culf A, Léger D, Chute IC, Belkaid A, Rise M, Gamperl AK, Hubert S, Kimball J, Ouellette RJ, Johnson SC, Bowman S, and Rise ML. 2011. Development and experimental validation of a 20K Atlantic cod (*Gadus morhua*) oligonucleotide microarray based on a collection of over 150,000 ESTs. Mar Biotechnol. 13 (4): 733–750.

Bowman S, Hubert S, Higgins B, Stone C, Kimball J, Borza T, Bussey JT, Simpson G, Hall JR, Hori TS, Feng CY, Gamperl AK, Booman M, Rise M, Symonds J, Johnson SC, and Rise ML. 2011. An integrated approach to gene discovery and marker development in Atlantic cod (*Gadus morhua*). Mar Biotechnol. 13 (2): 242–255.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, and Vingron M. 2001. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat Genet. 29: 365–371.

Canario AVM, Bargelloni L, Volckaert F, Houston RD, Massault C, and Guiguen Y. 2008. Genomics toolbox for farmed fish. Rev Fish Sci. 16 (S1): 3–15.

Cerdá J, Mercadé J, Lozano JJ, Manchado M, Tingaud-Sequeira A, Astola A, Infante C, Halm S, Viñas J, Castellana B, Asensio E, Cañavate P, Martínes-Rodríguez G, Piferrer F, Planas JV, Prat F, Yúfera M, Durany O, Subirada F, Rosell E, and Maes T. 2008. Genomic resources for a commercial flatfish, the Senegalese sole (*Solea senegalensis*): EST sequencing, oligo microarray design, and development of the Soleamold bioinformatic platform. BMC Genomics. 9: 508.

Chen LM, Wang F, Song W, and Hew CL. 2006. Temporal and differential gene expression of Singapore grouper iridovirus. J Gen Virol. 87: 2907–2915.

Chou MY, Hsiao CD, Chen SC, Chen IW, Liu ST, and Hwang PP. 2008. Effects of hypothermia on gene expression in zebrafish gills: upregulation in differentiation and function of ionocytes as compensatory responses. J Exp Biol. 211: 3077–3084.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, and Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 21: 3674–3676.

Darias MJ, Zambonino-Infante JL, Hugot K, Cahu CL, Mazurais D. 2008. Gene expression patterns during the larval development of European sea bass (*Dicentrarchus labrax*) by microarray analysis. Mar Biotechnol. 10: 416–428.

Davidson WS, Koop BF, Jones SJ, Iturra P, Vidal R, Mass A, Jonassen I, Lien S, and Omholt SW. 2010. Sequencing the genome of the Atlantic salmon (*Salmo salar*). Genome Biol. 11: 403.

de Hoon MJ, Imoto S, Nolan J, and Miyano S. 2004. Open source clustering software. Bioinformatics. 20: 1453–1454.

Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, and Lempicki RA. 2003. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. 4: R60.

Devlin RH, Sakhrani D, Tymchuk WE, Rise ML, and Goh B. 2009. Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (*Oncorhynchus kisutch*). Proc Natl Acad Sci USA. 106: 3047–3052.

Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, and Conklin BR. 2003. MAPPFinder: using Gene Ontology and GenMAPP to create a

global gene-expression profile from microarray data. Genome Biol 4: R7.

Douglas SE. 2006. Microarray studies of gene expression in fish. OMICS. 10: 474–489.

Douglas SE, Knickle LC, Williams J, Flight RM, and Reith ME. 2008. A first generation Atlantic halibut *Hippoglossus hippoglossus* (L.) microarray: application to developmental studies. J Fish Biol. 72: 2391–2406.

Edvardsen RB, Malde K, Mittelholzer C, Taranger GL, and Nilsen F. 2011. EST resources and establishment and validation of a 16k cDNA microarray from Atlantic cod (*Gadus morhua*). Comp Biochem Phys D. 6 (1): 23–30.

Efron B and Tibshirani R. 2007. On testing the significance of sets of genes. Ann Appl Stat. 1: 107–129.

Eisen MB, Spellman PT, Brown PO, and Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA. 95: 14863–14868.

Ewart KV, Belanger JC, Williams J, Karakach T, Penny S, Tsoi SCM, Richards RC, and Douglas SE. 2005. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Dev Comp Immunol. 29: 333–347.

Feng CY, Johnson SC, Hori TS, Rise M, Hall JR, Gamperl AK, Hubert S, Kimball J, Bowman S, and Rise ML. 2009. Identification and analysis of differentially expressed genes in the immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*. Physiol Genomics. 37: 149–163.

Feng CY and Rise ML. 2010. Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (*Gadus morhua*). Mol Immunol. 47: 763–784.

Ferraresso S, Vitulo N, Mininni AN, Romualdi C, Cardazzo B, Negrisolo E, Reinhardt R, Canario AVM, Patarnello T, and Bargelloni L. 2008. Development and validation of a gene expression oligo microarray for the gilthead sea bream (*Sparus aurata*). BMC Genomics. 9: 580.

Gahr SA, Rexroad CE, Rise ML, Hunt P, Koop B. 2007. A survey of expressed sequence tags from the rainbow trout (*Oncorhynchus mykiss*) pituitary. Anim Biotechnol. 18: 213–230.

Gahr SA, Vallejo RL, Weber GM, Shepherd BS, Silverstein JT, and Rexroad CE 3rd. 2008. Effects of short-term growth hormone treatment on liver and muscle transcriptomes in rainbow trout (*Oncorhynchus mykiss*). Physiol Genomics. 32: 380–392.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maecler M, Rossini AJ, Sawitzki G, Smith C, Tierney L, Yang JY, and Zhang J. 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5: R80.

Gerwick L, Corley-Smith G, and Bayne CJ. 2007. Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish Shellfish Immunol. 22: 157–171.

Goetz FW and MacKenzie S. 2008. Functional genomics with microarrays in fish biology and fisheries. Fish Fish. 9: 378–395.

Goetz FW, Rise ML, Rise M, Goetz GW, Binkowski F, and Shepherd BS. 2009. Stimulation of growth and changes in the hepatic transcriptome by 17β-estradiol in the yellow perch (*Perca flavescens*). Physiol Genomics. 38: 261–280.

Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J, and Conesa A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36: 3420–3435.

Govoroun M, Le Gac F, and Guiguen Y. 2006. Generation of a large scale repertoire of expressed sequence tags (ESTs from normalized rainbow trout cDNA libraries. BMC Genomics. 7: 196.

Gunnarsson L, Kristiansson E, Rutgersson C, Sturve J, Fick, J, Förlin L, and Larsson DG. 2009. Pharmaceutical industry effluent diluted 1:500 affects global gene expression, cytochrome P4501A activity and plasma phosphate in fish. Environ Toxicol Chem. 28: 2639–2647.

Hori TS, Gamperl AK, Afonso LOB, Johnson SC, Hubert S, Kimball J, Bowman S, and Rise ML. 2010. Heat-shock responsive genes identified and validated in Atlantic cod (*Gadus morhua*) liver, head kidney and skeletal muscle using genomic techniques. BMC Genomics. 11: 72.

Huang DW, Sherman BT, and Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 4: 44–57.

Jordal AEO, Torstensen BE, Tsoi S, Tocher D, Lall SP, and Douglas SE. 2005. Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). J Nutr. 135: 2355–2361.

Jørgensen SM, Afanasyev S, and Krasnov A. 2008. Gene expression analyses in Atlantic salmon

challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality. BMC Genomics. 9: 179.

Ju Z, Dunham RA, and Liu Z. 2002. Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation. Mol Genet Genomics. 268: 87–95.

Koop BF, von Schalburg KR, Leong J, Walker N, Lieph R, Cooper GA, Robb A, Beetz-Sargent M, Holt RA, Moore R, Brahmbhatt S, Rosner J, Rexroad CE 3rd, McGowan CR, and Davidson WS. 2008. A salmonid EST genomic study: genes, duplications, phylogeny and microarrays. BMC Genomics. 9: 545.

Krasnov A, Koskinen H, Pehkonen P, Rexroad CE, Afanasyev S, and Mölsä H. 2005. Gene expression in the brain and kidney of rainbow trout in response to handling stress. BMC Genomics. 6: 3.

Kurobe T, Yasuike M, Kimura T, Hirono I, and Aoki T. 2005. Expression profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. Dev Comp Immunol. 29: 515–523.

Leaver MJ, Villeneuve LA, Obach A, Jensen L, Bron JE, Tocher DR, and Taggart JB. 2008. Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). BMC Genomics. 9: 299.

Li RW and Waldbieser GC. 2006. Production and utilization of a high-density oligonucleotide microarray in channel catfish, *Ictalurus punctatus*. BMC Genomics. 7: 134.

Lie KK, Lanzen A, Breilid H, and Olsvik PA. 2009. Gene expression profiling in Atlantic cod (*Gadus morhua* L.) from two contaminated sites using a custom-made cDNA microarray. Environ Toxicol Chem. 28: 1711–1721.

Lu J, Peatman E, Yang Q, Wang S, Hu Z, Reecy J, Kucutas H, and Liu Z. 2011. The catfish genome database cBARBEL: an informatic platform for genome biology of ictalurid catfish. Nucleic Acids Res. 39 (Database issue): D815–D821.

MacKenzie S, Balasch JC, Novoa B, Ribas L, Roher N, Krasnov A, and Figueras A. 2008. Comparative analysis of the acute response of the trout, *O. mykiss*, head kidney to in vivo challenge with virulent and attenuated infectious hematopoietic necrosis virus and LPS-induced inflammation. BMC Genomics. 9: 141.

Majji S, Thodima V, Arnizaut A, Deng Y, May W, Sittman D, Waldbieser GC, Hanson L, Cuchens MA, Bengten E, and Chinchar VG. 2009. Expression profiles of cloned channel catfish (*Ictalurus punctatus*) lymphoid cell lines and mixed lymphocyte cultures. Dev Comp Immunol. 33: 224–234.

Malek RL, Sajadi H, Abraham J, Grundy M, and Gerhard GS. 2004. The effects of temperature reduction on gene expression and oxidative stress in skeletal muscle from adult zebrafish. Comp Biochem Phys C. 138: 363–373.

Martin SAM, Collet B, Mackenzie S, Evensen O, and Secombes CJ. 2008. Genomic tools for examining immune gene function in salmonid fish. Rev Fish Sci. 16 (S1): 112–118.

Martin SAM, Taggart JB, Seear P, Bron JE, Talbot R, Teale AJ, Sweeney GE, Høyheim B, Houlihan DF, Tocher DR, Zou J, and Secombes CJ. 2007. Interferon type I and type II responses in an Atlantic salmon (*Salmo salar*) SHK-1 cell line by the salmon TRAITS/SGP microarray. Physiol Genomics. 32: 33–44.

Matsuyama T, Fujiwara A, Nakayasu C, Kamaishi T, Oseko N, Hirono I, and Aoki T. 2007. Gene expression of leucocytes in vaccinated Japanese flounder (*Paralichthys olivaceus*) during the course of experimental infection with *Edwardsiella tarda*. Fish Shellfish Immunol. 22: 598–607.

Metzker ML. 2010. Sequencing technologies—the next generation. Nat Rev Genet. 11: 31–46.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A., Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstråle M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altschuler D, and Groop LC. 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 34: 267–273.

Morrison RN, Cooper GA, Koop BF, Rise ML, Bridle AR, Adams MB, and Nowak BF. 2006. Transcriptome profiling of the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.): a role for the tumor suppressor protein p52 in AGD-pathogenesis? Physiol Genomics. 26: 15–34.

Murray HM, Lall SP, Rajaselvam R, Boutilier LA, Flight RM, Blanchard B, Colombo S, Mohindra V, Yúfera M, and Douglas SE. 2010. Effect of early introduction of microencapsulated diet to larval Atlantic halibut, *Hippoglossus hippoglossus* L. assessed by microarray analysis. Mar Biotechnol. 12: 214–229.

Nakayama K, Kitamura SI, Murakami Y, Soung JY, Jung SJ, Oh MJ, Iwata H, and Tanabe S. 2008. Toxicogenomic analysis of immune system-related genes in Japanese flounder (*Paralichthys olivaceus*) exposed to heavy oil. Mar Pollut Bull. 57: 445–452.

Nash JH, Findlay WA, Luebbert CC, Mykytczuk OL, Foote SJ, Taboada EN, Carillo CD, Boyd JM, Colquhoun DJ, Reith ME, and Brown LL. 2006. Comparative genomics profiling of clinical isolates of *Aeromonas salmonicida* using DNA microarrays. BMC Genomics. 7: 43.

Olohan LA, Li W, Wulff T, Jarmer H, Gracey AY, and Cossins AR. 2008. Detection of anoxia-responsive genes in cultured cells of the rainbow trout *Oncorhynchus mykiss* (Walbaum), using an optimized, genome-wide oligoarray. J Fish Biol. 72: 2170–2186.

Palti Y. 2009. Aquaculture genomics. In: Molecular Research in Aquaculture (ed. Overturf K). Wiley-Blackwell, Ames, IA. pp 103–145.

Park KC, Osborne JA, Montes A, Dios S, Nerland AH, Novoa B, Figueras A, Brown L, Johnson SC. 2009. Immunological responses of turbot (*Psetta maxima*) to nodavirus infection or polyriboinosinic polyribocytidylic acid (pIC) stimulation, using expressed sequence tags (ESTs) analysis and cDNA microarrays. Fish Shellfish Immunol. 26: 91–108.

Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, Li P, Wang S, Somridhivej B, Dunham R, and Liu Z. 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Dev Comp Immunol. 31: 1183–1196.

Pei W, Noushmehr H, Costa J, Ouspenskaia MV, Elkahioun AG, and Feldman B. 2007. An early requirement for maternal FoxH1 during zebrafish gastrulation. Dev Biol. 310: 10–22.

Pollard KS, Dudoit S, and van der Laan MJ. 2005. Multiple testing procedures: the multtest package and applications to genomics. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor (eds. Gentleman R, Carey V, Dudoit S, Irizarry R, and Huber W). Springer, New York. pp 249–272.

Rawls JF, Samuel BS, and Gordon JI. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc Natl Acad Sci USA. 101: 4596–4601.

R Development Core Team. 2010. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Australia.

Rise ML, Douglas SE, Sakhrani D, Williams J, Ewart KV, Rise M, Davidson WS, Koop BF, and Devlin RH. 2006. Multiple microarray platforms utilized for hepatic gene expression profiling of growth hormone transgenic coho salmon with and without ration restriction. J Mol Endocrinol. 37: 259–282.

Rise ML, Hall J, Rise M, Hori T, Gamperl AK, Kimball J, Hubert S, Bowman S, and Johnson SC. 2008. Functional genomic analysis of the response of Atlantic cod (*Gadus morhua*) spleen to the viral mimic polyriboinosinic polyribocytidylic acid (pIC). Dev Comp Immunol. 32: 916–931.

Rise ML, Hall JR, Rise M, Hori TS, Browne M, Gamperl AK, Hubert S, Kimball J, Bowman S, and Johnson SC. 2010. Impact of asymptomatic nodavirus carrier state and intraperitoneal viral mimic injection on brain gene expression in Atlantic cod (*Gadus morhua*). Physiol Genomics. 42: 266–280.

Rise ML, Jones SRM, Brown GD, von Schalburg KR, Davidson WS, and Koop BF. 2004a. Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to *Piscirickettsia salmonis* infection. Physiol Genomics. 20: 21–35.

Rise ML, Liu ZJ, Douglas SE, Brown LL, Nash JHE, and McFall-Ngai MJ. 2009. Aquaculture-related applications of DNA microarray technology. In: Molecular Research in Aquaculture (ed. Overturf K). Wiley-Blackwell, Ames, IA. pp 63–101.

Rise ML, von Schalburg KR, Brown GD, Mawer MA, Devlin RH, Kuipers N, Busby M, Beetz-Sargent M, Alberto R, Gibbs AR, Hunt P, Shukin R, Zeznik JA, Nelson C, Jones SRM, Smailus DE, Jones SJM, Schein JE, Marra MA, Butterfield YSN, Stott JM, Ng SHS, Davidson WS, and Koop BF. 2004b. Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res. 14: 478–490.

Rise ML, von Schalburg KR, Cooper GA, and Koop BF. 2007. Salmonid DNA microarrays and other tools for functional genomics research. In: Aquaculture Genome Technologies (ed. Liu Z). Blackwell Publishing, Ames, IA. pp 369–411.

Saldanha AJ. 2004. Java Treeview—extensible visualization of microarray data. Bioinformatics. 20: 3246–3248.

Salem M, Kenney PB, Rexroad CE 3rd, and Yao J. 2008. Development of a 37k high-density oligonucleotide microarray: a new tool for functional genome research in rainbow trout. J Fish Biol. 72: 2187–2206.

Salomonis N, Hanspers K, Zambon AC, Vranizan K, Lawlor SC, Dahlquist KD, Doniger SW, Stuart J, Conklin BR, and Pico AR. 2007. GenMAPP 2: new features and resources for pathway analysis. BMC Bioinformatics. 8: 217.

Sarropoulou E, Kotoulas G, Power DM, and Geisler R. 2005. Gene expression profiling of gilthead sea bream during early development and detection of stress-related gene by the application of cDNA microarray technology. Physiol Genomics. 23: 182–191.

Schwender H, Krause A, and Ickstadt K. 2006. Identifying interesting genes with siggenes. RNews. 45–50.

Skugor S, Glover KA, Nilsen F, and Krasnov A. 2008. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*). BMC Genomics. 9: 498.

Smyth GK. 2005. Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor (eds. Gentleman R, Carey V, Dudoit S, Irizarry R, and Huber W). Springer, New York. pp 397–420.

Stockhammer OW, Zakrzewska A, Hegedus Z, Spaink HP, and Meijer AH. 2009. Transcriptome profiling and functional analysis of the zebrafish embryonic innate immune response to Salmonella infection. J Immunol. 182: 5641–5653.

Sturn A, Quackenbush J, and Trajanoski Z. 2002. Genesis: cluster analysis of microarray data. Bioinformatics. 18: 207–208.

Subramanian A, Kuehn H, Gould J, Tamayo P, and Mesirov JP. 2007. GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics. 23: 3251–3253.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gilette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, and Mesirov JP. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 102: 15545–15550.

Tilton SC, Gerwick LG, Hendricks JD, Rosato CS, Corley-Smith G, Givan SA, Bailey GS, Bayne CJ, and Williams DE. 2005. Use of a rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B1-induced hepatocellular carcinoma compared to adjacent liver. Toxicol Sci. 88: 319–330.

Tusher VG, Tibshirani R, and Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA. 98: 5116–5121.

van der Meer DLM, van den Thillart GEEJM, Witte F, de Bakker MAG, Besser J, Richardson MK, Spaink HP, Leito JTD, and Bagowski CP. 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. Am J Physiol–Reg I. 289: R1512–R1519.

von Schalburg KR, Rise ML, Cooper GA, Brown GD, Gibbs AR, Nelson CC, Davidson WS, and Koop BF. 2005. Fish and chips: various methodologies demonstrate utility of a 16,006-gene salmonid microarray. BMC Genomics. 6: 126.

Vornanen M, Hassinen M, Koskinen H, and Krasnov A. 2005. Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. Am J Physiol–Reg I. 289: R1177–R1184.

Wettenhall JM and Smyth GK. 2004. LimmaGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics. 20: 3705–3706.

Williams DR, Li W, Hughes MA, Gonzalez SF, Vernon C, Vidal MC, Jeney Z, Jeney G, Dixon P, McAndrew B, Bartfai R, Orban L, Trudeau V, Rogers J, Matthews L, Fraser EJ, Gracey AY, and Cossins AR. 2008. Genomic resources and microarrays for the common carp *Cyprinus carpio* L. J Fish Biol. 72: 2095–2117.

Wiseman S, Osachoff H, Bassett E, Malhotra J, Bruno J, Vanaggelen G, Mommsen TP, and Vijayan MM. 2007. Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. Comp Biochem Phys D. 2: 234–244.

Workenhe ST, Hori TS, Rise ML, Kibenge MJT, and Kibenge FSB. 2009. Infectious salmon anaemia virus (ISAV) isolates induce distinct gene expression responses in the Atlantic salmon (*Salmo salar*) macrophage/dendritic-like cell line TO, assessed using genomic techniques. Mol Immunol. 46: 2955–2974.

Yang YH and Paquet AC. 2005. Preprocessing two-color spotted arrays. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor (eds. Gentleman R, Carey V, Dudoit S, Irizarry R, and Huber W). Springer, New York. pp 49–70.

Young ND, Cooper GA, Nowak BF, Koop BF, and Morrison RN. 2008. Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.). Mol Immunol. 45: 2581–2597.

Chapter 2

Advances in Genomics and Genetics of Penaeid Shrimp

Javier Robalino, Robert W. Chapman, Enrique de la Vega, Nuala A. O'Leary, Danielle M. Gorbach, Zhi-Qiang Du, Max F. Rothschild, Craig L. Browdy, Gregory W. Warr, and Yannick Labreuche

INTRODUCTION

Penaeid shrimp constitute one of the most important groups of species for aquaculture worldwide, ranking second overall in value in 2006 (FAO 2007). Sustainable development of this industry could greatly benefit from progress in our basic knowledge of genetics, genomics, and molecular immunology of shrimp. Recently, the application of high-throughput molecular tools and approaches has led to significant developments in these fields. This chapter describes the current status of efforts to catalog the transcriptome of shrimp through the collection, curation, database development, and analyses of expressed sequence tags (ESTs) and through analysis of differential gene expression. Advances in the development of other genetic resources such as genetic maps and genomic libraries are also discussed. We emphasize how these tools can provide, and are providing, new molecular information about biological processes of relevance to shrimp aquaculture, such as immune responses and reproductive physiology. We then consider the contributions of reverse genetics through RNA-mediated interference (RNAi), which is now being used to test the involvement of specific genes in aquaculture-relevant traits. Proof-of-concept studies also demonstrate that RNAi is a promising approach to the development of antiviral therapies. Future developments in shrimp genetics and genomics that will further contribute to advancing biotechnological applications in aquaculture are also discussed throughout this chapter.

EST COLLECTION AS AN APPROACH TO GENE DISCOVERY IN SHRIMP

ESTs are short DNA sequences generated by large scale, single-pass sequencing of randomly picked cDNA clones from libraries. They generally represent a single tissue or condition of interest at a given time, thus providing a "snapshot" of the physiological status of a cell, tissue, or an organism. EST sequencing projects can provide an efficient and rapid means for discovering new genes, alleles, and polymorphisms, thereby providing data on gene expression and regulation, and for the development of genome maps.

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

Genomic research by EST analysis has been conducted for several shrimp species, including *Penaeus monodon*, *Fenneropenaeus chinensis*, *Marsupenaeus japonicus*, *Litopenaeus setiferus,* and *Litopenaeus vannamei* (Table 2.1). Most of these analyses have been small scale sequencing efforts conducted by individual laboratories studying mostly shrimp immunity and disease, and have contributed to the identification of a significant number of previously undescribed genes.

In contrast to small scale EST projects, when large sequencing efforts are conducted, the rate of return of novel genes rapidly decreases as sequencing progresses. This is caused by a small number of genes producing large quantities of mRNA and appearing as highly redundant in the EST clones being sequenced. Therefore, for this kind of initiative, it is recommended to use some form of normalization or redundancy subtraction to maximize the rate of gene discovery (Soares et al. 1994). We have used a direct redundancy subtraction method, which involves subtracting highly redundant genes from arrayed cDNA libraries (de la Vega and O'Leary, unpublished). Briefly, the process involves producing a typical cDNA library, which is then plated onto large agar plates with antibiotic selection, and single colonies are picked robotically into 384 well plates. The resulting library is robotically spotted at high density onto nitrocellulose membranes and direct redundancy subtraction is achieved by sequencing 384 samples and identifying the most redundant clones. These clones are then used to produce probes, which are hybridized to the membranes, highly redundant genes are thereby identified, and those clones that did not hybridize to the probes are then "cherry picked" and rearrayed. This whole process yields a low redundancy library, which increases the rate of gene discovery (Fig. 2.1). This approach has been used to sequence more than 150,000 ESTs from hemocytes, gills, hepatopancreas, lymphoid organ, ventral nerve cord, and eyestalk of *L. vannamei*, resulting in the identification of around 15,000 unigenes. All of these ESTs are publicly available at the NCBI EST database (http://www.ncbi.nlm.nih.gov/dbEST) or at the Marine Genomics website (www.marinegenomics.org). This and other efforts have contributed, as of January 2009, to a total of 179,032 ESTs for penaeid shrimp (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html).

Although the comprehensive annotation of the now abundant EST data available for *L. vannamei* is at a very early stage, it is expected to yield a large number of novel genes involved in immunity, respiration, endocrinology, and digestion, among other biological processes, providing an opportunity to better understand the physiology of shrimp. In addition, large numbers of molecular markers such as single nucleotide polymorphisms (SNPs) or microsatellite markers are expected to be mined out of these sequences, making them available for shrimp breeding programs interested in using marker-assisted selection. Some efforts in this respect have already been reported (see discussion later in this chapter) and show promise in the ability to use this EST information for marker development.

MEDIUM- TO HIGH-THROUGHPUT STUDIES OF DIFFERENTIAL EXPRESSION AND GENE DISCOVERY

While ESTs can provide a "snapshot" of gene expression in a tissue of interest, it is not a cost-effective approach to identifying differential gene expression in multiple samples. Differential expression is often of particular interest as a first approach to understanding gene function. The presumption that expression of a gene is largely restricted to the cell or tissue where it is needed, at the time when it is needed, provides the rationale for the significant attention given to this issue. In shrimp of aquaculture significance, medium- to high-throughput methods for assessing changes in gene expression have been most intensively applied to studying the response to pathogens and to stimulators of the immune system.

For species where the expected gene content vastly exceeds the number of known genes (which is the case in shrimp), methods of assessing changes in gene expression that do not rely on previously known sequence information are particularly useful. Two widely used methods of this kind, suppression subtractive hybridization (SSH; Diatchenko et al. 1996) and mRNA differential display (DD; Liang and Pardee 1992), are aimed at the isolation of cDNA fragments derived from differentially expressed genes. Comparative analyses of ESTs, isolated

P1: SFK/UKS
BLBS089-c02
P2: SFK
BLBS089-Fletcher
October 3, 2011
10:7
Trim: 246mm X 189mm
Printer Name: Yet to Come

Table 2.1. Studies Reporting Characterization of ESTs in Penaeid Shrimp.

Species	Tissues and Conditions Under Study	#ESTs/Unique Sequences	Principal Findings/Genes	Reference
Pm	Testes	896/NA	Discovery of genes involved in reproductive maturation and sex determination. Description of testes-specific genes.	Leelatanawit et al. 2009
Pm	Lymphoid organ from *Vibrio harveyi*-infected animals compared to noninfected animals	408 normal and 625 infected/NA	Discovery of genes differentially expressed in response to bacterial challenge. Discussion of the cathepsin family L and B. http://pmonodon.biotec.or.th/home.jsp	Pongsomboon et al. 2008b
Pm	Vitellogenic ovaries	1051/559	Identification of sex-related genes. Further analysis of chromobox protein (CBX), which is preferentially expressed in ovaries.	Preechaphol et al. 2007
Pm	Postlarvae infected with WSSV	6671 normal and 7298 infected/ 9622	Gene discovery in postlarvae shrimp. http://xbio.lifescience.ntu.edu.tw/pm/	Leu et al. 2007
Pm	Eyestalk, hepatopancreas, haematopoietic tissue, haemocyte, lymphoid organ, and ovary from normal, heat stressed, WSSV, YHV, and *V. harveyi*-infected shrimp	10,100/4845	Large scale gene discovery, GO analysis. http://p monadon.biotec.or.th	Tassanakajon et al. 2006
Pm	Hemocytes from *V. harveyi*-infected animals compared to noninfected animals	1062/NA	Immune gene discovery with a focus on antimicrobial peptides.	Supungul et al. 2004

(*Continued*)

Table 2.1. (*Continued*)

Species	Tissues and Conditions Under Study	#ESTs/Unique Sequences	Principal Findings/Genes	Reference
Fc	Cephalothorax	10,446/NA	Immune gene discovery. Immune genes including lectins, serine proteases, serpins, and lysozyme are discussed.	Shen et al. 2004
Pm	Hemocytes from noninfected animals	615/NA	Identification of immune genes expressed in normal hemocytes with focus on penaeidins, heat-shock proteins, and anti-LPS protein.	Supungul et al. 2002
Mj	Hemocytes from WSSV-infected animals compared to noninfected animals	635 normal and 370 WSSV/NA	Immune gene discovery	Rojtinnakorn et al. 2002
Lv, Lse	Hemocytes and hepatopancreas	2045/268	Immune gene discovery	Gross et al. 2001
Pm	Cephalothorax, eyestalk, and pleopod tissue	151/NA	Gene discovery with a discussion of tissue-specific genes.	Lehnert et al. 1999
Lv	Hemocytes, gills, hepatopancreas, lymphoid organ, eyestalk and ventral nerve cord	13,656/7466	Large-scale gene discovery with focus on genes from immune-related tissues. www.marinegenomics.org	O'Leary et al. 2006
Lv	Abdominal muscle	311/NA	Profile of gene expression in shrimp muscle tissue.	Cesar et al. 2008

Pm, *Penaeus monodon*; Fc, *Fenneropenaeus chinensis*; Mj, *Marsupenaeus japonicus*; Lv, *Litopenaeus vannamei*; Lse, *Litopenaeus setiferus*; NA, not reported.

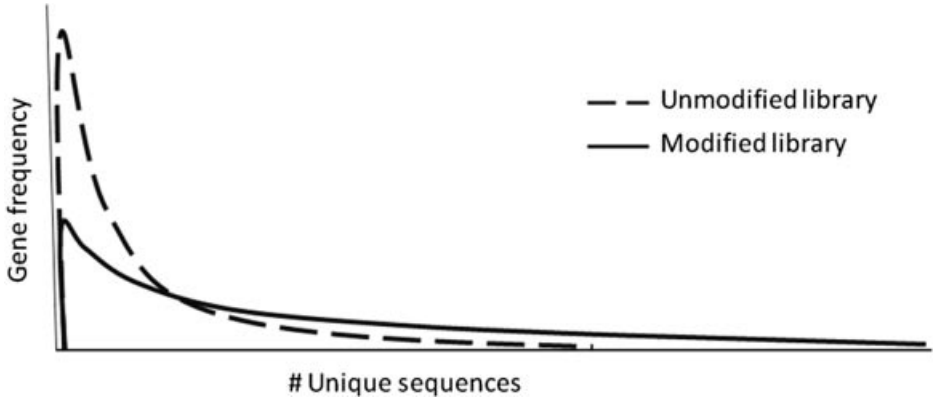


Figure 2.1. Expected gene frequency distribution in an unmodified vs. a depleted or modified cDNA library. Removing highly redundant gene sequences from a cDNA library results in a better rate of return of unique sequences with the same sequencing effort.

from tissues or cells subjected to conditions of interest, can also be used as a means of identifying genes potentially regulated by such conditions (see Table 2.1 for examples). All these methods are exploratory in nature, each subject to caveats and biases of their own, such that independent experimental confirmation of differential expression by direct methods (such as quantitative reverse-transcription PCR) is essential. SSH and DD are especially limited in terms of direct discovery of new genes in understudied species, because they involve the isolation of short cDNA fragments, rather than full-length cDNAs. SSH and DD are also less amenable than traditional EST mining to high-throughput sequencing, as the complexity of the cDNA pools obtained by these methods is generally low.

Processes of interest to shrimp aquaculture that have been studied using differential expression cloning approaches include gametogenesis, abiotic stress responses, and immune responses (Table 2.2). Many of the sequences isolated from these studies do not allow identification of genes with predictable homology or function. Presumably, some of these sequences correspond to novel genes, while others represent poorly conserved and/or short regions from otherwise conserved genes.

A markedly different approach to study differential gene expression at high-throughput is the use of microarray technology. Microarrays are arrangements of DNAs immobilized to solid supports in such a way that each element in the arrangement corresponds to a single known DNA sequence. Probing these arrangements with mRNA (or the corresponding cDNA) obtained from animals of interest allows a quantitative assessment of the expression of each gene whose sequence is represented on the microarray. Remarkably, thousands to millions of such DNA elements can be simultaneously evaluated due to the capacity of current technology to immobilize DNA on surfaces at extremely high densities. Microarrays with gene contents ranging from a few dozen to a few thousand genes have been generated and used to study the expression in *L. vannamei*, *P. monodon*, *L. stylirostris*, *M. japonicus*, and *F. chinensis*. In Table 2.3, some of these studies are summarized, in terms of the nature of the microarrays developed, and in terms of their most significant findings potentially relevant to aquaculture.

New gene discovery and the identification of differentially expressed genes provide only an initial step toward better understanding the shrimp biology and toward using such information to improve shrimp aquaculture. For almost every shrimp gene considered in the studies described in Tables 2.1, 2.2, and 2.3, the most critical experimental information necessary to understand its function is still

P1: SFK/UKS
BLBS089-c02
P2: SFK
BLBS089-Fletcher
October 3, 2011
10:7
Trim: 246mm X 189mm
Printer Name: Yet to Come

Table 2.2. Differential Expression Cloning Studies in Penaeid Shrimp.

Species	Tissues and Conditions Under Study	Method	# Unique Sequences Identified	Principal Findings	Reference
Ls	Hepatopancreas from shrimp experimentally infected with WSSV vs. uninfected shrimp	DD	32	One of the earliest indications of the challenges of assigning function to novel sequences in shrimp	Astrofsky et al. 2002
Pm	Hepatopancreas from shrimp surviving a WSSV outbreak vs. uninfected shrimp	DD	NA	PmAV, a C-type lectin with apparent antiviral activity in a heterologous (nonshrimp) virus–host system	Luo et al. 2003
Mj	Hemocytes from shrimp stimulated by heat-killed microbes vs. shrimp not stimulated	SSH	77 with homology	Diverse functional groups of genes appear regulated by heat-killed microbes, including protease inhibitors	He et al. 2004
Mj	Hemocytes from shrimp surviving a WSSV outbreak vs. uninfected shrimp	SSH	30	Diverse functional categories including chaperones, lectins, and protease inhibitors enriched in animals surviving an outbreak	He et al. 2005
Mj	Hepatopancreas from shrimp surviving a WSSV outbreak vs. uninfected shrimp	SSH	31	Diverse functional categories including lectins, glucan binding proteins, and small GTPases enriched in animals surviving an outbreak	Pan et al. 2005
Ls	Hemocytes from animals surviving a bacterial challenge compared to nonsurviving animals	SSH	184	Increased expression of several antimicrobials (e.g., penaeidin, lysozyme, cryptdin-like) co-relating with survival to bacterial infection	de Lorgeril et al. 2005
Pm	Hemocytes from *Vibrio harveyi*-infected animals compared to noninfected animals	DD	24 with homology	Induction of expected immune effectors (e.g., lysozyme, ALF, transglutaminase), and of Argonaute, a gene of the RNAi pathway	Somboonwiwat et al. 2006

Lv	Hemocytes, gills, or hepatopancres from shrimp induced with heat-inactivated microbes, dsRNA, or WSSV compared to mock-treated animals. Same tissues from shrimp infected with WSSV at permissive and nonpermissive temperatures	SSH	3231 from both SSH and standard cDNA libraries	A wide range of genes with potential roles in immunity, including antimicrobials, signaling factors, transcription factors, regulators of apoptosis, were suggested to be regulated by immune stimuli	Robalino et al. 2007
Lv	Hepatopancreas from uninfected members of a shrimp family selected based on its reduced susceptibility to WSSV compared to a family with high susceptibility	SSH	193, with 40 matches to known genes	Diverse functional categories of genes suggested to be enriched in WSSV-resistant family. These included lysozymes, cathepsins, lectins, and other potential antimicrobials	Zhao et al. 2007
Pm	Hemocytes from shrimp subjected to osmotic, hypoxic, or thermal stress, compared to nonstressed animals	SSH	176, with 58 matches to known genes	Some known immune factors and, strikingly, many retrotransposon-related sequences are regulated during abiotic stress	de la Vega et al. 2007a
Me	Hepatopancreas from shrimp at different stages of ovarian development	DD	15 clones with homology	Vitellogenin and some functionally diverse enzymes regulated during ovarian maturation	Wong et al. 2008
Pm	Testes from broodstock vs. juvenile shrimp	SSH	80 with homology	Discovery of a progestin receptor membrane component 1 gene	Leelatanawit et al. 2008

Ls, *Litopenaeus stylirostris*; Pm, *Penaeus monodon*; Mj, *Marsupenaeus japonicus*; Lv, *Litopenaeus vannamei*; Me, *Metapenaeus ensis*; DD, differential display; SSH, suppression subtractive hybridization; NA, not reported.

P1: SFK/UKS
BLBS089-c02
P2: SFK
BLBS089-Fletcher
October 3, 2011
10:7
Trim: 246mm X 189mm
Printer Name: Yet to Come

Table 2.3. Microarray Studies in Penaeid Shrimp.

Species	Estimated Gene Content (Unique Sequences)	Tissue and Conditions Studied	Principal Findings	Reference
Ls	84	Hepatopancreas from shrimp experimentally infected with WSSV vs. uninfected shrimp	Several potential pattern recognition proteins, such as lectins and LPS/glucan binding protein, induced by WSSV	Dhar et al. 2003
Fc	1578 unique cDNAs plus 1536 SSH clones	Whole cephalothorax from shrimp experimentally infected with WSSV vs. uninfected shrimp, and from naturally infected shrimp vs. uninfected wild animals	Diverse groups of genes regulated during experimental and natural WSSV infection, including chaperones and genes involved in metabolism and cell structure	Wang et al. 2006
Lv	2469	Hepatopancreas from shrimp experimentally infected with WSSV vs. uninfected shrimp	Induction of some known antimicrobials and repression of oxidative stress genes and of the immune transcription factor STAT	Robalino et al. 2007
Pm	NA	Hemocytes from shrimp exposed to either osmotic, hypoxic, or thermal stress	A complex response to abiotic stress, which included regulation of known immune factors (e.g., crustin, lysozyme, transglutaminase), as well as changes in mRNAs corresponding to retrotransposons	de la Vega et al. 2007b
Pm	2028	Hemocytes from YHV-infected shrimp vs. mock-infected animals	Known immune genes with complex patterns of temporal regulation. Cathepsin L highly induced in YHV-infected hemocytes	Pongsomboon et al. 2008a
Mj	2036	Hemocytes from peptidoglycan stimulated shrimp vs. animals not stimulated	Known immune factors such as antimicrobial proteins respond to peptidoglycan stimulation	Fagutao et al. 2008
Fc	3114 elements	Hepatopancreas, hemocytes, gills, and lymphoid organ from shrimp infected with WSSV vs. mock infected, and from animals stimulated with heat-killed *Vibrio anguillarum* vs. mock stimulated	Very functionally diverse groups of genes responsive to either immune stimulus, and also an overlapping response was observed	Wang et al. 2008a

Ls, *Litopenaeus stylirostris*; Fc, *Fenneropenaeus chinensis*; Lv, *Litopenaeus vannamei*; Pm, *Penaeus monodon*; Mj, *Marsupenaeus japonicus*; NA, not reported.

lacking. However, for some of the genes found to be regulated during immune challenge, functional follow-up studies have started to define their roles in the defense from pathogens (see next section on shrimp RNAi studies). These types of investigations can begin to elucidate immune pathways, improving our fundamental understanding of shrimp immunity. Furthermore, such genes represent good candidates for markers of disease resistance in breeding efforts and for the development of tools useful to monitor shrimp health in aquaculture settings. Thus, the discovery of genes that are differentially expressed in situations of interest, coupled with subsequent functional characterization of the gene products, provide important avenues to the development of biotechnological tools for aquaculture.

RNAi-BASED APPLICATIONS IN SHRIMP AQUACULTURE: FROM REVERSE GENETICS TO CONTROL OF DISEASES

First described in nematodes (Fire et al. 1998) and subsequently in most eukaryotes studied so far, RNAi is a highly conserved nucleic acid-based mechanism, mediating sequence-specific targeted gene silencing. This machinery is initiated by double-stranded RNA (dsRNA), which is processed by a Dicer family member into small effector RNA duplexes (e.g., siRNAs, miRNAs, esiRNAs, etc.). The small RNAs are incorporated onto a multimeric protein complex, the RNA-induced silencing complex (RISC) and related complexes, which mediate targeted degradation, translational repression, and other silencing phenomena by means of complementary base-pairing. Because dsRNA or short interfering RNAs (siRNAs) can be supplied exogenously to trigger specific gene silencing, RNAi has rapidly become the most widely used gene-silencing tool in a broad variety of eukaryotic organisms (reviewed in Campbell and Choy 2005). In this section, we review current RNAi-based research in shrimp and discuss future applications of this phenomenon in shrimp aquaculture.

RNAi Mechanisms in Shrimp

The existence of an intact RNAi machinery in shrimp was first proposed by Robalino and collaborators, who demonstrated in *L. vannamei* that in vivo administration of dsRNA induced a downregulation of endogenous or viral gene expression in a sequence-specific manner (Robalino et al. 2005). Additional evidence came from the identification of possible RNAi pathway components in the black tiger shrimp *P. monodon*: Pem-AGO (Dechklar et al. 2008); Pm-Ago, another member of the Argonaute protein family (Unajak et al. 2006); and a Dicer nuclease, Pm-Dcr1 (Su et al. 2008). However, more work will be necessary to define the roles (if any) played by each of these components in shrimp RNAi. Nevertheless, these results strongly suggest the existence of functional RNAi in shrimp and have opened the possibility, for the first time, of using reverse genetic approaches to understand gene function in these organisms.

Methods to Trigger Gene Silencing by RNAi in Shrimp

In shrimp, targeted gene silencing has been experimentally induced by several methods: in vivo injection, in vitro delivery to primary cell cultures, and feeding bacteria-carrying dsRNA in vivo.

Injection of dsRNA

Several studies have shown in different shrimp species the ability of ectopic dsRNA to spread from the site of injection (pereiopod, tail muscle) to distant tissues to mediate a potent depletion of cognate mRNAs, thus facilitating reliable and reproducible gene-silencing experiments (Table 2.4). However, several factors that are likely to affect silencing efficiency, such as target sequence selection and dsRNA dose, are still in need of detailed study in the different shrimp species. Altogether, injection of long dsRNA is to date the most widely used method for interrupting gene expression in shrimp in vivo.

Injections of siRNAs to induce knockdown of target gene expression have also been reported in shrimp (Table 2.4). Generated by cleavage of dsRNA by Dicer, siRNAs are 21∼25-mer duplexes that confer sequence specificity to the silencing complexes. In mammals, siRNAs are extensively used not only because of their great specificity and efficacy but also because these duplexes are small enough to bypass the induction of potent immune responses elicited by longer dsRNAs (Elbashir et al. 2001). In shrimp, important discrepancies in obtained results have been observed when using siRNAs in vivo (see Table 2.4).

Table 2.4. RNAi-Based Experiments in Penaeid Shrimp.

Species	Target Gene	RNAi-Based Application	Phenotype	References
Shrimp gene physiological function studies				
Pm	Argonaute (Pem-AGO)	dsRNA Transfection into Oka cells	Impaired RNAi ability	Dechklar et al. 2008
Lv	Putative farnesoic acid O-methyltransferase (LvFAMeT)	dsRNA Injection into the 5th pereiopod	Role in molting Lethal phenotype induced	Hui et al. 2008
Lsc	Crustacean hyperglycemic hormone (CHH)	dsRNA Injection into abdominal body cavity	Decrease in hemolymph glucose levels	Lugo et al. 2006
Lv	Putative ion transport peptide (LvITP)	dsRNA Injection into the 5th pereiopod	Role in osmo-regulatory function Lethal phenotype induced	Tiu et al. 2007
Lv	Hemocyanin	dsRNA/siRNA Intramuscular injection	– Reduction in hemocyanin mRNA levels after dsRNA injection – siRNAs failed to induce genetic interference	Robalino et al. 2005
Lv	CDP (CUB domain protein)	dsRNA Intramuscular injection	Reduction in CDP mRNA levels	Robalino et al. 2005
Me	Molt-inhibiting hormone (MeMIH-B)	dsRNA Injection into the pereiopod	Gonad-stimulatory function	Tiu and Chan 2007
Pm	Gonad-inhibiting hormone (Pem-GIH)	dsRNA – Injection into the pereiopod – Incubation in explant culture	Gonad-inhibitory function	Treerattrakool et al. 2008
Pm	Vitellogenin receptor (VgR)	dsRNA Intramuscular injection	Role in the processing of vitellogenin	Tiu et al. 2008

Host–pathogen interaction studies				
Lv	Anti-lipopolysaccharide factor (LvALF)	dsRNA Intramuscular injection	Role in immune function against bacterial and fungal infections	de la Vega et al. 2008
Lv	Crustin (LvABP1)	dsRNA Intramuscular injection	Role in antibacterial response	Shockey et al. 2009
Pm	Prophenoloxidases (*Pm*proPO1,2)	dsRNA Intramuscular injection	Role in antibacterial response	Amparyup et al. 2009
Mj	Rab-GTPase (PjRab)	siRNA Intramuscular injection	Increased viral replication	Wu et al. 2008
Pm	Small GTPase protein (PmRab7)	dsRNA Intramuscular injection	Role in the endosomal trafficking pathway	Ongvarrasopone et al. 2008
Mj	Transglutaminase (TGase) Clotting protein (CP)	dsRNA Intramuscular injection	Role in immune function against bacterial and fungal infections	Maningas et al. 2008
Mj	Caspase (PjCaspase)	siRNA Intramuscular injection	Role in virus-induced apoptosis	Wang et al. 2008b
Mj	β-integrin	siRNA Intramuscular injection	Cellular receptor for WSSV infection	Li et al. 2007
Lv	Caspase-3 homolog (Cap-3)	dsRNA Intramuscular injection	Role in virus-induced apoptosis	Rijiravanich et al. 2008
Pm	Dicer 1 (Pm Dcr1)	dsRNA Intramuscular injection	Increased susceptibility to viral infection	Su et al. 2008
Pm	YHV-binding protein (pmYRP65)	dsRNA Transfection into Oka cells	Inhibition of YHV infection	Assavalapsakul et al. 2006
RNAi-mediated antiviral silencing				
Fc	– VP28 (WSSV) – VP281 (WSSV) – protein kinase coding gene (WSSV)	dsRNA Intramuscular injection	Higher survival rates	Kim et al. 2007

(*Continued*)

Table 2.4. (*Continued*)

Species	Target Gene	RNAi-Based Application	Phenotype	References
Pm	– Helicase-coding gene (YHV) – Polymerase coding gene (YHV) – Protease coding gene (YHV) – gp116 (YHV) – gp64 (YHV)	dsRNA Transfection into Oka cells	Inhibition of YHV replication	Tirasophon et al. 2005
Pm	YHV-protease	dsRNA Intramuscular injection	Inhibition of YHV multiplication in infected shrimp	Tirasophon et al. 2007
Pm	Coding region of a protease gene (YHV)	dsRNA Intramuscular injection	– Inhibition of YHV replication – Protection from YHV infection	Yodmuang et al. 2006
Pm	– Vp28 (WSSV) – Vp15 (WSSV)	siRNA – Transfection in insect cells – Intramuscular injection	– Silencing of homologous genes in a heterologous expression system – siRNAs failed to induce sequence-specific antiviral immunity	Westenberg et al. 2005
Mj	Vp28 (WSSV)	siRNA Intramuscular injection	Reduction in viral DNA production of infected animals	Xu et al. 2007
Lv	Vp19 (WSSV)	dsRNA/siRNAs Intramuscular injection	– Higher survival rates after dsRNA injection – siRNAs failed to induce antiviral immunity	Robalino et al. 2005

Lv	– DNA polymerase (WSSV) – Ribonucleotide reductase small subunit (WSSV) – Thymidine kinase (WSSV) – Thymidylate kinase (WSSV) – Vp24 (WSSV) – Vp28 (WSSV)	siRNA Intramuscular injection	– Inhibition of WSSV replication – Suppression of selected WSSV gene expression – Higher survival rates	Wu et al. 2007
Lv	– Ribonucleotide reductase small subunit (WSSV) – DNA polymerase DP (WSSV) – ORF WSV252 (WSSV) – Vp28 (WSV)	dsRNA Intramuscular injection	Protection from WSSV infection	Robalino et al. 2005
Lv	Predicted protease gene (TSV)	dsRNA Intramuscular injection	Protection from TSV infection	Robalino et al. 2005
Pm	Vp28 (WSSV)	– Bacterially expressed dsRNA – Oral administration	Protection from WSSV infection	Sarathi et al. 2008b
Pm	Vp28 (WSSV)	– Bacterially expressed dsRNA – Intramuscular injection	Protection from WSSV infection	Sarathi et al. 2008a

Pm, *Penaeus monodon*; Lv, *Litopenaeus vannamei*; Lsc, *Litopenaeus schmitti*; Me, *Metapenaeus ensis*; Mj, *Marsupenaeus japonicus*; Fc, *Fenneropenaeus chinensis*.

Results ranging from no silencing at all to highly effective silencing have been reported. The existence of different mechanisms for uptake of siRNA, selection and design methods of target sequences for RNAi, or duration of siRNA activity in vivo have been hypothesized to explain these inconsistencies (Shekhar and Lu 2009). These issues warrant further investigation.

In Vitro Delivery to Primary Cell Cultures

Few studies have demonstrated in shrimp the application of RNAi in transfection experiments (Tirasophon et al. 2005; Assavalapsakul et al. 2006; Dechklar et al. 2008). Because of the unavailability of clonal long-term cell lines in marine invertebrates (Rinkevich 2005), gene-specific dsRNA transfection experiments have been performed into primary cultures of lymphoid (Oka) cells, leading to downregulation of endogenous or viral cognate mRNAs. Recently, Treerattrakool et al. described successful RNAi-mediated knockdown by dsRNA adjunction to *P. monodon* eyestalk ganglia and abdominal nerve cord explant cultures (Treerattrakool et al. 2008). These studies expand significantly the experimental capabilities for exploring gene function in shrimp, allowing the study of organ-specific phenomena outside of the context of the whole animal.

Feeding or Bacteria-Mediated RNAi

Establishing RNAi by bacterial feeding was first envisioned in *Caenorhabditis elegans* (Timmons and Fire 1998) and later in planarians (Newmark et al. 2003), plants (Tenllado et al. 2003), and insects (Walshe et al. 2009). In shrimp, progress has been made recently in the use of nonpathogenic bacteria to induce gene silencing. Sarathi and colleagues were the first to report the production of dsRNA using prokaryotic expression systems for RNAi purposes in *P. monodon* (Sarathi et al. 2008a). Later, these authors investigated in vivo whether bacterially expressed dsRNA derived from viral sequences could specifically interfere with viral infection following oral administration. Two different delivery approaches were tested: animals were fed with pellet feed coated either with inactivated bacteria containing overexpressed dsRNA or with bacterially expressed dsRNA entrapped onto chitosan nanoparticles. Both feeding methodologies were conclusive, the best results being obtained when feed was coated with inactivated bacteria expressing dsRNA (Sarathi et al. 2008b).

RNAi as a Tool to Unravel Gene Function In Vivo

As previously discussed, EST mining and differential expression cloning have been applied in shrimp to gain insight into gene function. Ultimately, however, experimental manipulation of expression is necessary to directly address issues of function. In shrimp, RNAi is to date the only reliable tool for this type of experimentation. An increasing number of studies have resolved, in the recent past, gene functions involved in moulting, osmoregulation, reproduction, or glucose metabolism in shrimp by using gene-specific dsRNA/siRNA technology (Table 2.4). Although gene silencing by dsRNA can complicate immunology studies in shrimp due to nonspecific stimulation of antiviral responses (Robalino et al. 2004), it is possible to perform carefully designed experiments to explore at least some aspects of their immune system (de la Vega et al. 2008; Shockey et al. 2009; Amparyup et al. 2009). These studies are of great relevance to biotechnology in aquaculture, as they pave the way to start defining the relationship between known genes and traits of commercial importance.

RNAi as a Tool to Promote Antiviral Protection in Shrimp

The spread of infections due to geographically widespread viruses has led to huge economic losses in the shrimp farming industry, threatening its economic viability and long-term sustainability in some regions of the world (Lightner and Redman 1998; Lightner et al. 2006). To control the occurrence and spread of viral diseases, new strategies for disease prevention, such as exposure to inactivated virus or viral proteins, are currently being developed (reviewed in Johnson et al. 2008). Because the RNAi machinery allows gene silencing in a highly sequence-specific manner with little or no risk of undesired off-target effects, injections of viral gene-specific dsRNA/siRNA into shrimp seem to be a more powerful and attractive tool to inhibit viral replication and/or protect shrimp from viral infections than by other methods. Indeed, this strategy

has proven to be effective against three unrelated viruses (Table 2.1; see Shekhar and Lu 2009 for a review). Furthermore, as previously discussed, feeding of dsRNA has already been proven as a feasible approach to block viral pathogenesis in vivo (Sarathi et al. 2008b), opening the door to the development of dsRNA-based treatments applicable at a commercial scale.

MARKERS, GENETIC MAPS, AND LARGE INSERT GENOMIC LIBRARIES IN SHRIMP

The development of genetic markers for breeding purposes has been an area of significant interest among shrimp researchers for several years. Several types of markers, including microsatellites, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), and randomly amplified polymorphic DNAs (RAPDs), have been developed, to different extents, in several penaeid species (for examples see Garcia et al. 1994; Xu et al. 1999; Wilson et al. 2002; Meehan et al. 2003). Here, we focus mostly on how the new genomic resources previously described in this chapter have provided new opportunities for the identification of SNPs. Some significant efforts in the generation of genetic maps with large numbers of markers of diverse types will also be mentioned. Finally, we briefly comment on the status of large DNA libraries for shrimp, a resource that will prove essential for the future of genomics in these species.

Single Nucleotide Polymorphisms

Panels of genetic markers have been developed for both *L. vannamei* and *P. monodon*, consisting primarily of microsatellites and AFLPs, although a limited number of SNPs have also been reported (Glenn et al. 2005). Little genomic data is currently available for shrimp species and most of the sequence data has originated from EST libraries, which commonly may contain many sequencing errors. Consequently, locating new polymorphisms is a slow and arduous task, which is best completed through the use of computer predictions or large-scale sequencing techniques. Because of the limited alternatives, the primary method for in silico prediction of SNPs has been comparison of ESTs displaying sequence similarity, despite the challenges of distinguishing sequencing errors from true base differences. One pipeline used for such predictions is to cluster the available ESTs with CAP3 (Huang and Madan 1999) and predict SNPs using SNPidentifier (Gorbach et al. 2009), which has built-in quality control measures to compensate for the inherent errors in EST sequences. Thus far, this method has produced the most validated SNPs in shrimp (768 in *L. vannamei*) of any published process. When the same method was tried using ESTs from nine other shrimp species and validated in vivo only in *L. vannamei*, the success rate of SNP prediction was decreased significantly (from 44% to 11%; Gorbach et al., 2010).

A smaller-scale SNP identification method that has also been utilized is sequencing candidate genes. For a gene that is suspected to play a role in an important phenotype, primers are designed to sequence a region of the gene. When successful, one or more SNPs can be identified within the sequenced region. This process has identified at least 12 SNPs in *L. vannamei* and at least 3 SNPs in *P. monodon* (Glenn et al. 2005; Yu et al. 2006; Zeng et al. 2008). One of the SNPs discovered using this process has been placed on the *P. monodon* linkage map (Maneeruttanarungroj et al. 2006). Furthermore, sequences from various other resources can also be used to identify SNPs, e.g., short tandem repeats and AFLPs.

With the development of next-generation sequencing platforms, such as the Roche Genome Sequencer FLX™ system, Illumina Genome Analyzer™, and Applied Biosystems SOLiD™ sequencing system, large-scale SNP discovery has become extremely fast and efficient, for a lower cost. Currently, none of these technologies have been applied to shrimp, but this is certainly the future for genomics in general and therefore where shrimp genomics should go in the ensuing years.

Genetic Maps

Linkage maps based on microsatellite and AFLP markers have been published for both *L. vannamei* (Zhang et al. 2007) and *P. monodon* (Maneeruttanarungroj et al. 2006). A more complete linkage map for *P. monodon* has been constructed from AFLP markers (Staelens et al. 2008). This map identified some 44 male and 43 female linkage groups and, given the number of shrimp chromosomes (44; Chow et al. 1990), is likely to include the majority

of chromosomal linkage groups in this species. A new linkage map for *L. vannamei* is currently under construction (Du et al., unpublished), which incorporates a large number of SNP markers, and may be further extended with the output from next-generation sequencing technology.

Large Insert Size Genomic Libraries

Since the development of cloning methods for large DNA segments (O'Connor et al. 1989), bacterial artificial chromosomes (BACs) have become the preferred means for construction of libraries containing most, if not all, of the genome. Usually BACs contain inserts of size 150–350 kbp, but can be as large as 750 kbp. There are several reasons for constructing such large insert libraries, which include reduced overlap of individual clones, reduction in the number of clones that must be maintained for complete genome coverage, efficiencies in DNA sequencing and genome mapping, and source material for the creation of transgenic organisms. Unfortunately, generating a stable large insert BAC library for shrimp has proven to be difficult. Several mid-sized (50 kbp) fosmid libraries have, however, been generated and at least one of these is publicly available (http://www.genome.clemson.edu/). While these tools are available, they have not been exploited and, thus, great opportunities are available to the community in this area.

ANALYTICAL CHALLENGES IN GENOMICS AND GENETICS OF SHRIMP

A well-described characteristic of genomics studies is the generation of massively paralleled data sets, which will pose unprecedented analytical challenges. The well-known problems are how to manage large quantities of data and mine these data for significant biologically meaningful signals (Warr et al. 2007). These are issues for all genomics studies regardless of the species or the research area of interest, and the topic is frequently reviewed in the literature. Rather than rehash the issue, we chose to discuss a different topic that may be able to address some problems specific to shrimp aquaculture and potentially revise the way selective breeding in cultured species is prosecuted.

It is well known that phenotypic selection for quantitative traits in shrimp is hampered by the low observed heritabilities of certain desired traits (Perez-Rostro and Ibarra 2003) as opposed to typical values in agriculture species. This observation is consistent with what one might expect in a species, e.g., shrimp, which do not have a long history of domestication and inbreeding. For a trait like growth, which is likely influenced by a large number of genes and the environment, the absence of a long history of domestication reduces the probability that many loci will have gone to fixation by chance alone. This in turn makes it more difficult to detect the influence of individual genes that remain polymorphic in the population.

The high dimensionality of massive genomics data imposes a heavy burden on the analytical methods. How does one identify the important genetic markers when thousands to millions of genetic markers are available and only a small number of them are necessary for maximal information content? This is identical to the dimensionality reduction problem encountered in microarray analysis. Traditional linear algebra approaches (e.g., ANOVA) can only be useful for a limited number of markers and cannot predict the phenotype when there are nonlinear effects among alleles at a single locus or multigene interactions, which is almost certainly the case for most quantitative traits (cf. Brockmann et al. 2000; Liu et al. 2007; Han et al. 2008; Ankra-Badu et al. 2009 for examples).

Different statistical and computational methods have been proposed to address the issue of dimension reduction, for instance, Bayesian information criteria and penalized likelihood (cf. Manichaikul et al. 2008), with the general conclusion that the penalized LOD score approach provides an accurate selection of QTLs and their interactions, at least in low-dimensional space. Though promising, the approach taken by Manichaikul et al. (2008) is a rather brute force method that does not explore the global state space and could be more easily accomplished with artificial intelligence (AI) tools such as genetic algorithms and genetic programming optimization of neural networks (GPNN; Ritchie et al. 2003; Motsinger et al. 2006). Artificial neural networks (ANNs) and support vector machines (SVMs) have also been used for microarray and genotyping data (we note that SVMs using sigmoidal kernels are equivalent to a single layer ANN). The general

conclusion from studies employing the AI tools is that ANNs can detect the influence of multiple genetic and environmental factors on phenotypic traits but can be computationally expensive as a dimensionality reduction tool. In our own work, SVMs using polynomial kernels have been more efficient in dimensionality reduction than ANNs, but ANNs generally provide superior classifications and models of system dynamics (Chapman, unpublished). Furthermore, advances in analytical methods in other species that are already using genomic selection are likely to be applicable for improving breeding systems in aquaculture species.

CONCLUDING REMARKS

The last decade of research and development in shrimp genomics and genetics has seen significant advancements. An abundance of sequence information from expressed genes is available in public databases, providing a first glimpse at the gene content of several penaeid species. Researchers and aquaculture geneticists are only now beginning to exploit these resources, especially for the identification of genetic markers, candidate disease resistance genes, and genes linked to reproduction and other aquaculture-relevant processes. A growing number of initiatives are mining the available sequence data to implement markers and generate increasingly more extensive linkage maps. The number of shrimp genes for which at least some sequence information is now known is in the thousands, rather than the dozens, as was the case just a few years ago. The tools are also in place to refine the selection of candidate aquaculture-relevant genes through the characterization of two key aspects of the function of a gene: its expression and its loss-of-function phenotype. We can now measure the expression of thousands of these genes simultaneously, thanks to progress in transcriptomic methodologies, although much effort is still necessary within the community to meet the analytical challenges involved. Remarkably, it is now possible to block the expression of a gene in a targeted manner in vivo (using RNAi), a task that just a few years ago seemed only feasible in a few well-established animal models. A significant step forward in the control of viral diseases, one of the most important constraints to shrimp aquaculture, seems just around the corner. It is now possible to afford almost complete protection from highly pathogenic viruses by delivering dsRNA molecules that are produced by relatively simple methods.

So, what is the future of shrimp genetics and genomics as it relates to aquaculture? We propose that future success in this area is closely linked to the ability of the community to work cooperatively, sharing information and resources in benefit of the overall advancement of the science. The relative lack of unified efforts and multilab resources such as EST databases, microarray platforms, and library repositories, are indicators of the need to strengthen the ties among shrimp researchers. The challenges imposed by the sheer magnitude of genomic projects will likely be met only by a unified community, with a demonstrated capacity to gain the most out of shared resources. Such an evolution of attitudes will likely make it feasible to support a full-genome sequencing project for a penaeid shrimp in the near future.

ACKNOWLEDGMENTS

This material is based in part on work supported by the National Science Foundation. Any opinion, finding, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

REFERENCES

Amparyup P, Charoensapsri W, and Tassanakajon A. 2009. Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*. Dev Comp Immunol. 33(2): 247–256.

Ankra-Badu GA, Pomp D, Shriner D, Allison DB, and Yi N. 2009. Genetic influences on growth and body composition in mice: multilocus interactions. Int J Obes (Lond). 33(1): 89–95.

Assavalapsakul W, Smith DR, and Panyim S. 2006. Identification and characterization of a *Penaeus monodon* lymphoid cell-expressed receptor for the yellow head virus. J Virol. 80(1): 262–269.

Astrofsky KM, Roux MM, Klimpel KR, Fox JG, and Dhar AK. 2002. Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). Arch Virol. 147(9): 1799–1812.

Brockmann GA, Kratzsch J, Haley CS, Renne U, Schwerin M, and Karle S. 2000. Single QTL effects, epistasis, and pleiotropy account for two-thirds of the phenotypic F(2) variance of growth and obesity in DU6i x DBA/2 mice. Genome Res. 10(12): 1941–1957.

Campbell TN and Choy FY. 2005. RNA interference: past, present and future. Curr Issues Mol Biol. 7(1): 1–6.

Cesar JR, Zhao B, and Yang J. 2008. Analysis of expressed sequence tags from abdominal muscle cDNA library of the pacific white shrimp Litopenaeus vannamei. Animal. 2(9): 1377–1383.

Chow S, Dougherty WJ, and Sandifer PA. 1990. Meiotic chromosome complements and nuclear DNA contents of four species of shrimps of the genus *Penaeus*. J Crustacean Biol. 10(1): 29–36.

Dechklar M, Udomkit A, and Panyim S. 2008. Characterization of Argonaute cDNA from *Penaeus monodon* and implication of its role in RNA interference. Biochem Biophys Res Commun. 367(4): 768–774.

de la Vega E, Degnan BM, Hall MR, and Wilson KJ. 2007a. Differential expression of immune-related genes and transposable elements in black tiger shrimp (*Penaeus monodon*) exposed to a range of environmental stressors. Fish Shellfish Immunol. 23(5): 1072–1088.

de la Vega E, Hall MR, Wilson KJ, Reverter A, Woods RG, and Degnan BM. 2007b. Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. Physiol Genomics. 31(1): 126–138.

de la Vega E, O'Leary NA, Shockey JE, Robalino J, Payne C, Browdy CL, Warr GM, and Gross PS. 2008. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Mol Immunol. 45(7): 1916–1925.

de Lorgeril J, Saulnier D, Janech MG, Gueguen Y, and Bachere E. 2005. Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*. Physiol Genomics. 21(2): 174–183.

Dhar AK, Dettori A, Roux MM, Klimpel KR, and Read B. 2003. Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with white spot syndrome virus by cDNA microarrays. Arch Virol. 148(12): 2381–2396.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, and Siebert PD. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA. 93(12): 6025–6030.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411(6836): 494–498.

Fagutao FF, Yasuike M, Caipang CM, Kondo H, Hirono I, Takahashi Y, and Aoki T. 2008. Gene expression profile of hemocytes of kuruma shrimp, *Marsupenaeus japonicus* following peptidoglycan stimulation. Mar Biotechnol (NY). 10(6): 731–740.

FAO. 2007. The State of World Fisheries and Aquaculture 2006. FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations, Rome, Italy. 180pp.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, and Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391(6669): 806–811.

Garcia DK, Faggart MA, Rhoades L, Alcivar-Warren AA, Wyban JA, Carr WH, Sweeney JN, and Ebert KM. 1994. Genetic diversity of cultured *Penaeus vannamei* shrimp using three molecular genetic techniques. Mol Mar Biol Biotechnol. 3(5): 270–280.

Glenn KL, Grapes L, Suwanasopee T, Harris DL, Li Y, Wilson K, and Rothschild MF. 2005. SNP analysis of AMY2 and CTSL genes in *Litopenaeus vannamei* and *Penaeus monodon* shrimp. Anim Genet. 36(3): 235–236.

Gorbach DM, Hu Z-L, Du Z-Q, and Rothschild MF. 2009. SNP discovery in Litopenaeus vannamei with a new computational pipeline. Anim Genet. 40(1): 106–109.

Gorbach DM, Hu Z-L, Du Z-Q, and Rothschild MF. 2010. Mining ESTs to determine the usefulness of SNPs across shrimp species. Anim Biotechnol. 21(2): 100–103.

Gross PS, Bartlett TC, Browdy CL, Chapman RW, and Warr GW. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Dev Comp Immunol. 25(7): 565–577.

Han Y, Teng W, Sun D, Du Y, Qiu L, Xu X, and Li W. 2008. Impact of epistasis and QTL x environment interaction on the accumulation of seed mass of soybean (Glycine max L. Merr). Genet Res. 90(6): 481–491.

He N, Liu H, and Xu X. 2004. Identification of genes involved in the response of haemocytes of *Penaeus japonicus* by suppression subtractive hybridization (SSH) following microbial challenge. Fish Shellfish Immunol. 17(2): 121–128.

He N, Qin Q, and Xu X. 2005. Differential profile of genes expressed in hemocytes of White Spot Syndrome Virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. Antiviral Res. 66(1): 39–45.

Huang X and Madan A. 1999. CAP3: A DNA sequence assembly program. Genome Res. 9(9): 868–877.

Hui JH, Tobe SS, and Chan SM. 2008. Characterization of the putative farnesoic acid O-methyltransferase (LvFAMeT) cDNA from white shrimp, *Litopenaeus vannamei*: evidence for its role in molting. Peptides. 29(2): 252–260.

Johnson KN, van Hulten MC, and Barnes AC. 2008. "Vaccination" of shrimp against viral pathogens: phenomenology and underlying mechanisms. Vaccine. 26(38): 4885–4892.

Kim CS, Kosuke Z, Nam YK, Kim SK, and Kim KH. 2007. Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish Shellfish Immunol. 23(1): 242–246.

Leelatanawit R, Klinbunga S, Aoki T, Hirono I, Valyasevi R, and Menasveta P. 2008. Suppression subtractive hybridization (SSH) for isolation and characterization of genes related to testicular development in the giant tiger shrimp *Penaeus monodon*. BMB Rep. 41(11): 796–802.

Leelatanawit R, Sittikankeaw K, Yocawibun P, Klinbunga S, Roytrakul S, Aoki T, Hirono I, and Menasveta P. 2009. Identification, characterization and expression of sex-related genes in testes of the giant tiger shrimp *Penaeus monodon*. Comp Biochem Physiol A Mol Integr Physiol. 152(1): 66–76.

Lehnert SA, Wilson KJ, Byrne K, and Moore SS. 1999. Tissue-specific expressed sequence tags from the Black Tiger Shrimp *Penaeus monodon*. Mar Biotechnol (NY). 1(5): 465–476.

Leu JH, Chang CC, Wu JL, Hsu CW, Hirono I, Aoki T, Juan HF, Lo CF, Kou GH, and Huang HC. 2007. Comparative analysis of differentially expressed genes in normal and white spot syndrome virus infected *Penaeus monodon*. BMC Genomics. 8: 120.

Li DF, Zhang MC, Yang HJ, Zhu YB, and Xu X. 2007. Beta-integrin mediates WSSV infection. Virology. 368(1): 122–132.

Liang P and Pardee AB. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science. 257(5072): 967–971.

Lightner DV, Poulos BT, Tang-Nelson KF, Pantoja CR, Nunan LM, Navarro SA, Redman RM, and Mohney LL. 2006. Application of molecular diagnostic methods to penaeid shrimp diseases: advances of the past 10 years for control of viral diseases in farmed shrimp. Dev Biol. (Basel) 126: 117–122; discussion 325–326.

Lightner DV and Redman RM. 1998. Shrimp diseases and current diagnostic methods. Aquaculture. 164: 201–220.

Liu G, Yang J, Xu H, and Zhu J. 2007. Influence of epistasis and QTL x environment interaction on heading date of rice (*Oryza sativa* L.). J Genet Genomics. 34(7): 608–615.

Lugo JM, Morera Y, Rodriguez T, Huberman A, Ramos L, and Estrada MP. 2006. Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique. FEBS J. 273(24): 5669–5677.

Luo T, Zhang X, Shao Z, and Xu X. 2003. PmAV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. FEBS Lett. 551(1–3): 53–57.

Maneeruttanarungroj C, Pongsomboon S, Wuthisuthimethavee S, Klinbunga S, Wilson KJ, Swan J, Li Y, Whan V, Chu KH, Li CP, Tong J, Glenn K, Rothschild M, Jerry D, and Tassanakajon A. 2006. Development of polymorphic expressed sequence tag-derived microsatellites for the extension of the genetic linkage map of the black tiger shrimp (*Penaeus monodon*). Anim Genet. 37(4): 363–368.

Manichaikul A, Moon JY, Sen S, Yandell BS, and Broman KW. 2008. A model selection approach for the identification of quantitative trait loci in experimental crosses, allowing epistasis. Genetics. 181(3): 1077–1086.

Maningas MB, Kondo H, Hirono I, Saito-Taki T, and Aoki T. 2008. Essential function of transglutaminase and clotting protein in shrimp immunity. Mol Immunol. 45(5): 1269–1275.

Meehan D, Xu Z, Zuniga G, and Alcivar-Warren A. 2003. High frequency and large number of polymorphic microsatellites in cultured shrimp, *Penaeus* (*Litopenaeus*) *vannamei* (Crustacea: Decapoda). Mar Biotechnol (NY). 5(4): 311–330.

Motsinger AA, Lee SL, Mellick G, and Ritchie MD. 2006. GPNN: power studies and applications of a neural network method for detecting gene–gene interactions in studies of human disease. BMC Bioinformatics. 7: 39.

Newmark PA, Reddien PW, Cebria F, and Sanchez Alvarado A. 2003. Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. Proc Natl Acad Sci USA. 100(Suppl 1): 11861–11865.

O'Connor M, Peifer M, and Bender W. 1989. Construction of large DNA segments in *Escherichia coli*. Science. 244(4910): 1307–1312.

O'Leary NA, Trent HF III, Robalino J, Peck MET, McKillen DJ, and Gross PS. 2006. Analysis of multiple tissue-specific cDNA libraries from the Pacific whiteleg shrimp, *Litopenaeus vannamei*. Integrative and Comparative Biology. 46(6): 931–939.

Ongvarrasopone C, Chanasakulniyom M, Sritunyalucksana K, and Panyim S. 2008. Suppression of PmRab7 by dsRNA inhibits WSSV or YHV infection in shrimp. Mar Biotechnol (NY). 10(4): 374–381.

Pan D, He N, Yang Z, Liu H, and Xu X. 2005. Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. Dev Comp Immunol. 29(2): 103–112.

Perez-Rostro CI and Ibarra AM. 2003. Heritabilities and genetic correlations of size traits at harvest size in sexually dimorphic Pacific white shrimp (*Litopenaeus vannamei*) grown in two environments. Aquac Res. 34: 1079–1085.

Pongsomboon S, Tang S, Boonda S, Aoki T, Hirono I, Yasuike M, and Tassanakajon A. 2008a. Differentially expressed genes in *Penaeus monodon* hemocytes following infection with yellow head virus. BMB Rep. 41(9): 670–677.

Pongsomboon S, Wongpanya R, Tang S, Chalorsrikul A, and Tassanakajon A. 2008b. Abundantly expressed transcripts in the lymphoid organ of the black tiger shrimp, *Penaeus monodon*, and their implication in immune function. Fish Shellfish Immunol. 25(5): 485–493.

Preechaphol R, Leelatanawit R, Sittikankeaw K, Klinbunga S, Khamnamtong B, Puanglarp N, and Menasveta P. 2007. Expressed sequence tag analysis for identification and characterization of sex-related genes in the giant tiger shrimp *Penaeus monodon*. J Biochem Mol Biol. 40(4): 501–510.

Rijiravanich A, Browdy CL, and Withyachumnarnkul B. 2008. Knocking down caspase-3 by RNAi reduces mortality in Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* challenged with a low dose of white-spot syndrome virus. Fish Shellfish Immunol. 24(3): 308–313.

Rinkevich B. 2005. Marine invertebrate cell cultures: new millennium trends. Mar Biotechnol (NY). 7(5): 429–439.

Ritchie MD, White BC, Parker JS, Hahn LW, and Moore JH. 2003. Optimization of neural network architecture using genetic programming improves detection and modeling of gene–gene interactions in studies of human diseases. BMC Bioinformatics. 4: 28.

Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, and Warr G. 2004. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol. 78(19): 10442–10448.

Robalino J, Bartlett T, Shepard E, Prior S, Jaramillo G, Scura E, Chapman RW, Gross PS, Browdy CL, and Warr GW. 2005. Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J Virol. 79(21): 13561–13571.

Robalino J, Almeida JS, McKillen D, Colglazier J, Trent HF, 3rd, Chen YA, Peck ME, Browdy CL, Chapman RW, Warr GW, and Gross PS. 2007. Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. Physiol Genomics. 29(1): 44–56.

Rojtinnakorn J, Hirono I, Itami T, Takahashi Y, and Aoki T. 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. Fish Shellfish Immunol. 13(1): 69–83.

Sarathi M, Simon MC, Ahmed VP, Kumar SR, and Hameed AS. 2008a. Silencing VP28 gene of white spot syndrome virus of shrimp by bacterially expressed dsRNA. Mar Biotechnol (NY). 10(2): 198–206.

Sarathi M, Simon MC, Venkatesan C, and Hameed AS. 2008b. Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot syndrome virus. Mar Biotechnol (NY). 10(3): 242–249.

Shekhar MS and Lu Y. 2009. Application of nucleic-acid-based therapeutics for viral infections in shrimp aquaculture. Mar Biotechnol (NY). 11(1): 1–9.

Shen YQ, Xiang JH, Wang B, Li FH and Tong W. 2004. Discovery of immune-related factors in

Fenneropenaeus chinensis by annotation of ESTs. Prog Nat Sci. 14(1): 47–54.

Shockey JE, O'Leary NA, de la Vega E, Browdy CL, Baatz JE, and Gross PS. 2009. The role of crustins in *Litopenaeus vannamei* in response to infection with shrimp pathogens: an in vivo approach. Dev Comp Immunol. 33(5): 668–673.

Soares MB, Bonaldo MF, Jelene P, Su L, Lawton L, and Efstratiadis A. 1994. Construction and characterization of a normalized cDNA library. Proc Natl Acad Sci USA. 91(20): 9228–9232.

Somboonwiwat K, Supungul P, Rimphanitchayakit V, Aoki T, Hirono I, and Tassanakajon A. 2006. Differentially expressed genes in hemocytes of *Vibrio harveyi*-challenged shrimp *Penaeus monodon*. J Biochem Mol Biol. 39(1): 26–36.

Staelens J, Rombaut D, Vercauteren I, Argue B, Benzie J, and Vuylsteke M. 2008. High-density linkage maps and sex-linked markers for the black tiger shrimp (*Penaeus monodon*). Genetics. 179(2): 917–925.

Su J, Oanh DT, Lyons RE, Leeton L, van Hulten MC, Tan SH, Song L, Rajendran KV, and Walker PJ. 2008. A key gene of the RNA interference pathway in the black tiger shrimp, *Penaeus monodon*: identification and functional characterisation of Dicer-1. Fish Shellfish Immunol. 24(2): 223–233.

Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, and Tassanakajon A. 2004. Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. Dis Aquat Organ. 61(1–2): 123–135.

Supungul P, Klinbunga S, Pichyangkura R, Jitrapakdee S, Hirono I, Aoki T, and Tassanakajon A. 2002. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). Mar Biotechnol (NY). 4(5): 487–494.

Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, Sritunyalucksana K, Phongdara A, Pongsomboon S, Supungul P, Tang S, Kuphanumart K, Pichyangkura R, and Lursinsap C. 2006. *Penaeus monodon* gene discovery project: the generation of an EST collection and establishment of a database. Gene. 384: 104–112.

Tenllado F, Martinez-Garcia B, Vargas M, and Diaz-Ruiz JR. 2003. Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. BMC Biotechnol. 3: 3.

Timmons L and Fire A. 1998. Specific interference by ingested dsRNA. Nature. 395(6705): 854.

Tirasophon W, Roshorm Y, and Panyim S. 2005. Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochem Biophys Res Commun. 334(1): 102–107.

Tirasophon W, Yodmuang S, Chinnirunvong W, Plongthongkum N, and Panyim S. 2007. Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antiviral Res. 74(2): 150–155.

Tiu SH and Chan SM. 2007. The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone from the shrimp *Metapenaeus ensis*. FEBS J. 274(17): 4385–4395.

Tiu SH, Benzie J, and Chan SM. 2008. From hepatopancreas to ovary: molecular characterization of a shrimp vitellogenin receptor involved in the processing of vitellogenin. Biol Reprod. 79(1): 66–74.

Tiu SH, He JG, and Chan SM. 2007. The LvCHH-ITP gene of the shrimp (*Litopenaeus vannamei*) produces a widely expressed putative ion transport peptide (LvITP) for osmo-regulation. Gene. 396(2): 226–235.

Treerattrakool S, Panyim S, Chan SM, Withyachumnarnkul B, and Udomkit A. 2008. Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS J. 275(5): 970–980.

Unajak S, Boonsaeng V, and Jitrapakdee S. 2006. Isolation and characterization of cDNA encoding Argonaute, a component of RNA silencing in shrimp (*Penaeus monodon*). Comp Biochem Physiol B Biochem Mol Biol. 145(2): 179–187.

Walshe DP, Lehane SM, Lehane MJ, and Haines LR. 2009. Prolonged gene knockdown in the tsetse fly Glossina by feeding double stranded RNA. Insect Mol Biol. 18(1): 11–19.

Wang B, Li F, Dong B, Zhang X, Zhang C, and Xiang J. 2006. Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray. Mar Biotechnol (NY). 8(5): 491–500.

Wang B, Li F, Luan W, Xie Y, Zhang C, Luo Z, Gui L, Yan H, and Xiang J. 2008a. Comparison of gene expression profiles of *Fenneropenaeus chinensis* challenged with WSSV and Vibrio. Mar Biotechnol (NY). 10(6): 664–675.

Wang L, Zhi B, Wu W, and Zhang X. 2008b. Requirement for shrimp caspase in apoptosis against virus infection. Dev Comp Immunol. 32(6): 706–715.

Warr GW, Almeida JS, and Chapman RW. 2007. Computational challenges for the analysis of large datasets related to aquatic environmental genomics. In: Aquaculture Genome Technologies (ed. Liu J). Blackwell Publishing, Oxford. pp. 413–426.

Westenberg M, Heinhuis B, Zuidema D, and Vlak JM. 2005. siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. Virus Res. 114(1–2): 133–139.

Wilson K, Li Y, Whan V, Lehnert S, Byrne K, Moore S, Pongsomboon S, Tassanakajon A, Rosenberg G, Ballment E, Fayazia Z, Swan J, Kenwaya M, and Benzie J. 2002. Genetic mapping of the black tiger shrimp *Penaeus monodon* with amplified fragment length polymorphism. Aquaculture. 204: 297–309.

Wong QW, Mak WY, and Chu KH. 2008. Differential gene expression in hepatopancreas of the shrimp *Metapenaeus ensis* during ovarian maturation. Mar Biotechnol (NY). 10(1): 91–98.

Wu Y, Lu L, Yang LS, Weng SP, Chan SM, and He JG. 2007. Inhibition of white spot syndrome virus in *Litopenaeus vannamei* shrimp by sequence-specific siRNA. Aquaculture. 271: 21–30.

Wu W, Zong R, Xu J, and Zhang X. 2008. Antiviral phagocytosis is regulated by a novel Rab-dependent complex in shrimp *Penaeus japonicus*. J Proteome Res. 7(1): 424–431.

Xu Z, Dhar AK, Wyrzykowski J, and Alcivar-Warren A. 1999. Identification of abundant and informative microsatellites from shrimp (*Penaeus monodon*) genome. Anim Genet. 30(2): 150–156.

Xu J, Han F, and Zhang X. 2007. Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. Antiviral Res. 73(2): 126–131.

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, and Panyim S. 2006. YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. Biochem Biophys Res Commun. 341(2): 351–356.

Yu M, Cheng Y, and Rothschild M. 2006. SNP analysis of molting related genes in *Penaeus monodon* and *Litopenaeus vannamei* shrimp. Arch Tierz, Dummerstorf. 49(4): 411–412.

Zeng D, Chen X, Li Y, Peng M, Ma N, Jiang W, Yang C, and Li M. 2008. Analysis of Hsp70 in *Litopenaeus vannamei* and detection of SNPs. J Crustacean Biol. 28(4): 727–730.

Zhang L, Yang C, Zhang Y, Li L, Zhang X, Zhang Q, and Xiang J. 2007. A genetic linkage map of Pacific white shrimp (*Litopenaeus vannamei*): sex-linked microsatellite markers and high recombination rates. Genetica. 131(1): 37–49.

Zhao ZY, Yin ZX, Weng SP, Guan HJ, Li SD, Xing K, Chan SM, and He JG. 2007. Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridisation. Fish Shellfish Immunol. 22(5): 520–534.

Chapter 3

Genetic and Genomic Approaches to Atlantic Halibut Broodstock Management

Michael Reith, Darrin Reid, Debbie Martin-Robichaud, and Tillmann Benfey

INTRODUCTION

In the early 1980s, Atlantic halibut was identified as a promising marine fish species to aid in the diversification of finfish aquaculture in temperate waters. Researchers in Norway, Scotland, Canada, and Iceland made significant progress to overcome difficulties related to early life history traits that confounded the development of culture protocols. Significant progress was made on improving spawning protocols and egg quality, increasing survival during the extensive yolk sac stage, improving larval and juvenile rearing protocols and nutrition, and determining factors that affect successful metamorphosis. By the mid-1990s, the feasibility of halibut aquaculture was well established (Olsen et al. 1999; Brown 2002).

At this point, researchers began considering issues related to increasing the efficiency of production to ensure the economic viability of the halibut aquaculture industry. A domesticated broodstock selected for production in a hatchery setting would certainly enhance production. Initially, all halibut hatcheries used wild-caught broodstock as the basis for their production. However, the establishment of broodstock was constrained by the need for extensive facilities to accommodate sizeable holding tanks due to the large size (>2 m, >200 kg) of the fish, in addition to the logistics of catching and transporting live, wild broodstock. For these reasons, production of cultured Atlantic halibut was usually founded on a relatively small number of wild broodstock. For example, the breeding stock for the initial production of juveniles in Atlantic Canada was based on 13 males and 14 females (Jackson et al. 2003).

In recognition of the need for a selected, domesticated broodstock, F1s from production crosses were maintained and reared to maturity. However, these fish were genetic and phenotypic unknowns due to constraints imposed by biology and hatchery management. Atlantic halibut are iteroparous batch spawners, ovulating eggs about every 4 days (Norberg et al. 1991). Ovulatory cycles are carefully monitored and eggs, which are stripped manually, are usually fertilized with milt from multiple males

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

to ensure fertilization success and increased genetic mixing. Initially, egg quality was highly variable, and crosses were based on gamete quality and availability; no other selection criteria were applied. To meet production and volume efficiencies, hatchery protocols required that yolk sac larvae from multiple egg batches be reared communally. For these reasons, pedigree tracking was essentially impossible, resulting in potential F1 broodstock with unknown parentage.

A further issue for broodstock selection is the long time to maturity for halibut, which takes 5–7 years, although several more years are required (about 10 years in total) until consistent spawning of high-quality gametes is achieved. This process thus requires significant financial and space resources for holding and maintaining substantial numbers of future broodstock.

A second opportunity for increasing halibut aquaculture production stems from the fact that Atlantic halibut females grow faster and mature at a larger size than males (Jákupsstovu and Haug 1988; Björnsson 1995). Currently, monosex stocks of many teleost species are commercially reared (Devlin and Nagahama 2002), but species-specific protocols employing various genetic, endocrine, or environmental manipulations must be developed to establish monosex lines.

To begin to implement these methods for improvement of halibut production, the development of substantial genetic resources for halibut would be required. Beginning in the late 1990s, a series of projects were implemented for improvement of halibut broodstock in Atlantic Canada (Fig. 3.1). In addition to those that will be described in this chapter, important resources for investigating halibut biology and gene expression such as EST sequences (Douglas et al. 2007) and a microarray (Douglas et al. 2008) have been developed. Altogether, these tools provide many of the resources needed for the enhancement of halibut production and the long-term growth of the industry.

PRODUCTION OF ALL-FEMALE STOCKS OF ATLANTIC HALIBUT

Like many flatfish species, halibut show sexually dimorphic size variation with females growing faster and attaining a larger size than males (Jákupsstovu and Haug 1988; Björnsson 1995). Recent commercial rearing of Atlantic halibut juveniles in sea cages in Eastern Canada has shown that the growth trajectory of male and female juvenile halibut diverges at about 600–800 g, with females becoming 20% larger than males (Imsland and Jonassen 2005; Power 2009). An obvious method for increasing production and economic return for halibut aquaculture would be to raise all-female stocks.

There are several options for generating all-female fish strains, due to the potential to manipulate fish gonad development with external factors. Temperature treatments applied during gonadal development have been shown to affect male/female ratios in a variety of fish (see Baroiller and D'Cotta 2001; Godwin et al. 2003 for review). However, this has not been found to be an effective approach for halibut (van Nes and Andersen 2006; Hughes et al. 2008). Alternatively, production of all-female populations can be achieved by direct or indirect feminization through steroid hormone treatments (Piferrer 2001). Direct feminization entails the application of

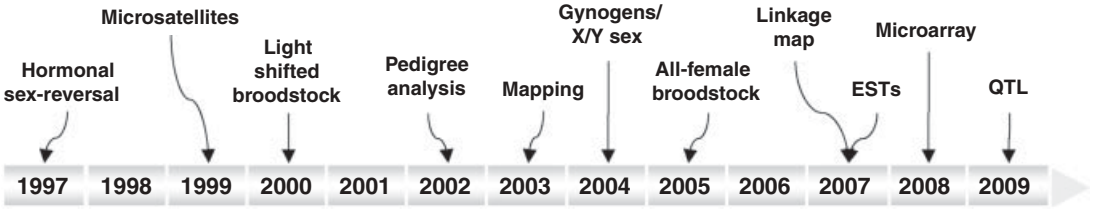


Figure 3.1. Timeline of the development of genetic/genomic tools and production improvements in Atlantic halibut in Atlantic Canada.

estrogen to sexually undifferentiated larvae to direct gonadal development into ovaries. However, direct sex reversal is generally avoided as a means of producing fish intended for market due to the exposure of fish to steroid hormones.

Indirect feminization is the preferred approach although it requires multiple generations. This method relies on the use of androgens to masculinize genotypic females so that they develop as phenotypic males ("neomales") and then crossing them with normal females to produce all-female stocks. This approach is possible when the mechanism of sex determination is female homogametic (analogous to the mammalian XX-female, XY-male system). This approach is well documented for salmonid fishes and used extensively to produce all-female populations of rainbow trout (Devlin and Nagahama 2002).

Sex Determination and Gynogenesis

The first step in using indirect feminization for the production of single-sex fish populations is to understand the genetic basis of sex determination. Simple systems of female homogamety or heterogamety (analogous to the avian WZ-female/ZZ-male system), which would be amenable to indirect feminization, have been demonstrated in some fish species, while complex systems occur in others (Devlin and Nagahama 2002; Nagahama 2005; Penman and Piferrer 2008). A straightforward approach to establishing whether fish have either of the simple sex-determining systems is to determine the sex ratio of gynogenetic populations (see Benfey 2009 for review).

Gynogenesis refers to development in the absence of the paternal genome. Gynogenetic fish ("gynogens") are produced by activating embryonic development in eggs using sperm containing DNA that cannot be replicated because it has been treated with radiation or chemicals, or because it comes from a different species. Ultraviolet radiation is commonly used for this purpose, requiring a dose sufficiently high to cause DNA cross-linking, but sufficiently low so as not to diminish sperm motility. Atlantic halibut gynogens can be produced from eggs activated by sperm diluted 1:80 in halibut seminal plasma and then exposed to 65 mJ/cm^2 of UV-radiation (Tvedt et al. 2006). With no further treatment, such gynogens are haploid. Diploidy is restored by exposing eggs activated with UV-treated sperm to thermal or hydrostatic pressure treatments soon after activation to block either the completion of meiosis (to retain the haploid second polar body) or the first mitotic cleavage. Hydrostatic pressure treatment of 58,600 kPa for 5 minutes, beginning 15 minutes postactivation at 5–6°C, has been used to produce gynogenetic diploid populations of Atlantic halibut (Tvedt et al. 2006). Genotyping (see below) confirmed the presence of only maternal alleles in the gynogens. The fact that these fish all developed as females confirmed female homogamety as the sex determining mechanism for halibut (Tvedt et al. 2006) and indicated the potential for indirect feminization in this species.

Gonadal Differentiation and Endocrine Sex Reversal

To carry out indirect feminization, conditions for the masculinization of females by exogenous application of sex steroids must be developed. Estrogens and androgens are the natural sex inducers in fish, directing gonadal differentiation toward ovaries or testes, respectively (Devlin and Nagahama 2002). The natural estrogen, 17β-estradiol (E2), and synthetic androgens, such as 17α-methyltestosterone (MT) and the nonaromatizable 17α-methyldihydrotestosterone (MDHT), are most commonly used for sex control in teleosts, although aromatase inhibitors, such as fadrozole, can also be used to masculinize fish as they block the conversion of testosterone to E2 by aromatase (Piferrer et al. 1994).

Critical to achieving sex reversal by the application of exogenous hormones is to introduce them during the species-specific labile period when undifferentiated gonads are most susceptible to endocrine influence. In halibut, gonadal sex differentiation occurs by 38.0 mm fork length, which coincides with postmetamorphic settling (Hendry et al. 2002). Feeding pelleted food containing 1 or 5 ppm MDHT to Atlantic halibut juveniles for 45 days beginning when they were 30.0 mm fork length resulted in populations that were 97–100% males, and feeding food treated with 10 ppm E_2 for the same duration resulted in 70–74% females (Hendry et al. 2003).

Since androgen treatment is applied to fish populations with both sexes, the resulting phenotypic males are a mixture of genetic males and females. To confirm the genetic sex of these phenotypic males, each one must be crossed with a normal female and the sex of the offspring determined. If the sire is a sex-reversed genotypic female ("neomale") then in species with female homogamety (XX), like Atlantic halibut, all offspring will be female. The phenotypic males produced by Hendry et al. (2003) were reared to maturity and all of those tested produced milt normally. Several of these fish were found to be neomales and one commercial Atlantic halibut hatchery has been using them to produce all-female stocks. Efforts to produce additional neomales are ongoing. The performance of the all-female stocks relative to normal stocks is in the process of being assessed. If, as expected, these stocks produce larger, faster growing fish, they will provide significant economic benefits to the halibut aquaculture industry.

PEDIGREE ANALYSIS

The current husbandry practices of most halibut hatcheries result in significant losses of information regarding the genetics and performance of their fish. During a halibut production run, several batches of fertilized eggs from multiple parents are combined and then further combined at the yolk-sac stage. No facility we are aware of consistently maintains single-family tanks in halibut aquaculture. While fertilization rates may be determined for individual crosses, other performance measures, such as size or survival, are usually determined only for an entire cohort or tank. A second factor affecting halibut production is the relatively small number of broodstock used, due to the high costs of maintaining these large fish. This increases the risk of inbreeding and the potential for loss of genetic diversity in subsequent generations. These constraints make the selection of better performing fish considerably more difficult as well as increasing the potential for genetic bottlenecks in future generations of broodstock.

A genetic pedigree that can unambiguously assign individuals to a single cross is a simple, yet powerful, tool to compensate for the constraints of halibut production. A pedigree allows hatcheries to

1. determine number and sizes of sib groups in a given F1 cohort,
2. determine success rate of crosses made,
3. determine survival rate of fertilized eggs from a given parent,
4. identify crosses/parents producing the largest or smallest fish,
5. assess genetic variation in the broodstock,
6. identify potential relatedness of founding broodstock,
7. begin a focused selective breeding program,
8. eliminate uncontrolled inbreeding, and
9. identify errors in spawning records.

Genetic markers suitable for pedigree analysis include microsatellites, restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), and single nucleotide polymorphisms (SNP). In aquaculture, microsatellites, which are short tandem nucleotide repeats that are codominantly expressed and highly polymorphic (Liu 1998), are the most commonly used genetic marker (Chistiakov et al. 2006). In Atlantic halibut, over 150 microsatellites have been developed (McGowan and Reith 1999; Coughlan et al. 2000; Reid et al. 2005; Reid et al. 2007) and microsatellites from several other flatfish species have been found to amplify well in halibut (McGowan and Reith 1999; Reid et al. 2007). Small numbers (5–7) of microsatellite markers have been shown to be sufficient to assign parentage to more than 90% of halibut progeny (Jackson et al. 2003; Frank-Lawale 2006).

The initial concern of most halibut pedigree studies is the effect on genetic variability (allele number, allelic diversity, observed, and expected heterozygosity) in going from wild broodstock to F1s (Jackson et al. 2003; Cross et al. 2005; Frank-Lawale 2006). Frank-Lawale (2006) identified a decrease in effective allele number, allelic diversity, and observed and expected heterozygosity in F1s. Cross et al. (2005) also noted a dramatic drop in allele number in halibut F1s. Jackson et al. (2003) identified decreases in allele number, heterozygosity, and effective population size (from 27 in the parental group to 13 in the F1s). Effective population size is essentially a measure of inbreeding in a small population that may have unequal sex ratios, nonrandom family sizes and/or nonrandom mating

(Falconer and MacKay 1996), all of which are true in an aquaculture setting. These decreases in genetic variability are to be expected in the process of domestication, but need to be monitored to ensure that genetic bottlenecks are not introduced, which ultimately constrain production.

Results from pedigree analyses provide several interesting observations regarding cross representation and family structure in halibut aquaculture production. The summary of halibut pedigree analyses shown in Table 3.1 includes both small (<150) and large (>250) groups of progeny selected as potential broodstock between 1995 and 2006. One notable observation is that most of the crosses attempted in a spawning season are not present in the selected progeny. In Table 3.1, the percentage of crosses present range from 11% (Nova Scotia 2005-2) to 35% (New Brunswick 1996-1), except for two year classes (Maine 2006-2, 2006-3) with 50% representation, although these cohorts contained only small 2×2 crosses.

Linked to this observation is the finding that certain crosses are heavily overrepresented in the selected progeny. In two instances, full-sib families make up the majority of progeny (Maine 2006-1, 2006-2), but more typically they represent 20–50% of the selected fish. Contributions by individual parents tend to be even greater, with a single dam or sire producing more than 50% of the selected offspring. Interestingly, these numbers seem to be fairly consistent even for large sample sizes (>500) and throughout the time period investigated. Clearly, certain families thrive much better than others, although it is not yet apparent whether their advantage is due to genetic or environmental factors (e.g., egg quality, feed quality, etc.).

Pedigree analysis also identifies errors in the spawning records. The majority of the pedigrees in Table 3.1 identified parents that had not been noted in the records. In some cases, these included parents for which a DNA sample was not available, so that the parental genotype had to be reconstructed (Frank-Lawale 2006; Reid, unpublished). There are obviously difficulties in maintaining accurate spawning records at hatcheries, perhaps due to inexact methods of marking broodstock.

Given the consistent overrepresentation of some crosses and the absence of the majority of crosses in a spawning season cohort, a pedigree analysis of future halibut broodstock is essential. With a single parent usually contributing to more than 50% of the progeny in a year class, the likelihood of randomly choosing two half-sib F1s to cross is high and that of choosing two full-sibs is substantial. A pedigree analysis will allow hatchery managers to confirm spawning records and to avoid future inbreeding.

HALIBUT GENETIC LINKAGE MAP

A genetic linkage map is a relatively quick and inexpensive way to develop a basic road map of an organism's genome. Linkage mapping relies on the observations that the amount of meiotic recombination between linked genetic markers approximates the physical distance between those markers. However, the frequency of recombination can vary within the genome, particularly at recombination hotspots, where the increased frequency of double-stranded DNA breaks leads to excess recombination. As well, sex-specific differences in recombination have been observed in a variety of animals (Hedrick 2007). Differences in recombination between the sexes are thought to arise from differences in synaptonemal complex formation in oocytes and spermatocytes (Kondo et al. 2001; Lynn et al. 2005). Genetic linkage maps thus serve as only an approximation of the genome.

The main utility of genetic maps arises from the fact that both phenotypic (i.e., physical traits or characteristics) and genetic (i.e., microsatellite, RFLP, AFLP, etc.) markers can be placed on a genetic map. This allows the identification of genetic markers that are linked to the phenotype and that may serve as a surrogate means of detecting the phenotype of interest or that serve as reference points for genome walking to identify the gene responsible for that phenotype. Genetic maps are also useful for the construction of a physical map based on large-insert clones such as bacterial or yeast artificial chromosomes. In addition, a well-defined genetic map is essential for robust quantitative trait locus (QTL) mapping to define markers associated with polygenic traits of economic interest (see below). A recent summary of the current status of fish linkage maps is available in Table 10.2 of Danzmann and Gharbi (2007).

A genetic linkage map for Atlantic halibut was constructed using 92 offspring from each of two

P1: SFK/UKS
BLBS089-c03
P2: SFK
BLBS089-Fletcher
October 3, 2011
10:10
Trim: 246mm X 189mm
Printer Name: Yet to Come

Table 3.1. Halibut Broodstock Pedigree Analysis.

	# Dams	# Sires	# Potential Crosses	# Crosses Detected	# F1s Genotyped	# F1s Assigned to a Single Cross	# Assigned to Unexpected Parents	Largest Full-Sib Family (%)*	Largest Half-Sib Family (%) M/F†
New Brunswick Hatchery (Jackson et al. 2003)									
1996-1	14	13	80	28	145	145	0	32 (22.1%)	34 (23.4%) F
Scottish Hatchery (Frank-Lawale 2006)									
1995	16	17	NR‡	17	270	229	6	56 (24.5%)	112 (49%) F
1998	21	17	NR	35	532	499	56	215 (43.1%)	380 (77%) M
Maine Hatchery (unpublished)									
2006-1	2	3	6	2	49	49	30	30 (61.2%)	30
2006-2	2	2	4	2	50	50	0	49 (98%)	49
2006-3	2	2	4	2	50	50	34	15 (30%)	26 (52%) M
Nova Scotia Hatchery (unpublished)									
2003-2	26	20	201	27	326	259	6	91 (35.1%)	147 (56.7%) F
2005-2	20	21	210	24	523	389	65	153 (39.3%)	241 (61.9%) M
2006-1	20	10	139	21	468	406	35	208 (51.2%)	339 (83.5%) F

*Number of progeny in the largest full-sib family and percentage of the assigned F1s that family represents.
†Number of progeny produced by a single dam or sire, percentage of the assigned F1s they represent, and whether the parent was male or female.
‡Crosses attempted were not recorded.

half-sib families (Reid et al. 2007). The genetic markers for mapping consisted of 476 microsatellite markers from halibut and related flatfish as well as 64 AFLP primer combinations. The resulting halibut linkage map contains 258 microsatellite and 346 AFLP markers. These genetic markers clustered into 24 linkage groups, consistent with the 24 chromosomes in the halibut karyotype (Brown et al. 1997). Separate maps were generated for the male and female parents, with the total map distances equal to 1459.6 cM and 1562.2 cM, respectively. The average spacing between markers was 3.5 cM and 4.3 cM in the male and female maps, respectively. The only significant difference in map arrangement between the male and female maps was a discontinuity in the male map in linkage group AH-13, which splits it into two smaller regions that are still colinear with the female map. This discontinuity in the male map appears to represent a recombination hotspot.

Gynogenetic diploids ($n = 46$) from an unrelated female halibut were used to estimate the location of the centromere on 19 of the 24 linkage groups (Reid et al. 2007). Nearly all linkage groups appeared to have acrocentric or telocentric centromere locations except for AH-20, where the centromere appears to map 20–30 cM from one end of the linkage group. These results are also consistent with the chromosome organizations visualized in the karyotype (Brown et al. 1997).

In addition to providing an outline of the genome, the halibut linkage map reveals significant differences in recombination between the sexes. Overall, recombination in the female parent is approximately twice that of the male parents. However, this overall difference in recombination rate varies considerably within linkage groups. Using the predicted centromere positions, linkage groups were split into centromeric and telomeric halves and the recombination rates in the two halves were compared between the sexes in each of the mapping families. In the centromeric half, the recombination rate in the female was 17.5 and 11 times that of each male, while the males had 3.1 and 2.6 times the recombination of the female in the telomeric half. Thus, in the female parents, recombination was much more frequent near the centromeres, while in the male parents, recombination tended to occur more often nearer the telomeres. These differences can be useful for determining marker order in the sex where the recombination frequency is high in the region of interest.

QUANTITATIVE TRAIT LOCI

Most of the physiological traits that are important to the aquaculture industry, such as growth or disease resistance, are quantitative in nature, meaning that they have a continuous distribution of phenotypic values. These quantitative traits are controlled by many genes, each with only a limited effect on the trait. The regions within the genome where these genes are located are referred to as QTL. QTL mapping (Tanksley 1993) is the process of identifying these genomic regions through statistical analysis. It provides information on both the location and number of contributing genes (MacKay 2001). QTL for economically important traits such as growth, temperature tolerance, disease resistance, and spawning time have been identified in many aquaculture species (see Table 11.1 in Korol et al. 2007).

Considerations for Halibut QTL Analysis

Halibut provide great opportunities and challenges for the experimental design of QTL studies. Because of their high fecundity and fully controlled breeding, any necessary crosses can be made during a spawning cycle and very large sib groups can be formed. Experimental size is only limited by the costs associated with tagging, genotyping, and phenotyping individuals. The current genetic map provides an average spacing between microsatellites (the preferred marker for QTL studies) of 7–8 cM, which should provide sufficient power for QTL resolution even though outbred populations will be used (Kolbehdari et al. 2005; Massault et al. 2008).

However, the long generation time of halibut (about 10 years to reach consistent reproduction) limits the type of crosses that can be made and extends the duration of many experiments. The long generation time means that simple genetic tools such as a backcross are currently unavailable and thus halibut QTL mapping experiments will have to use outbred populations. Outbred populations are limiting in that not all markers will be heterozygous, QTL alleles are incompletely known, families may or may not be segregating for the same QTL, and the relationship between linkage phase and the QTL alleles

is unknown (Falconer and MacKay 1996). Other factors affecting the power of QTL analysis are that the success of individual crosses is highly variable and that hatchery space constraints require that experimental populations be mixed in with production runs. While these factors constrain the sensitivity of QTL mapping experiments, attention to experimental design and appropriate statistical analyses (e.g., Massault et al. 2008) will allow sufficient power for QTL detection.

Status of QTL Mapping in Halibut

A preliminary QTL study has been conducted in Atlantic halibut based on trait data collected from the genetic mapping families at 61 days after first feeding (Reid et al. 2007). Significant QTL were identified for body size, pigmentation, metamorphosis, and eye migration (Reid et al., in prep). As well, experimental crosses have been established for a halibut growth QTL study, the results of which will be included in a broodstock selection program. Of course, QTL identification is only part of a complete selection program.

BROODSTOCK SELECTION

As halibut aquaculture production methods become more routine, the next step is to focus on the selection of future broodstock that outperform their wild parents. Generally, undomesticated wild fish do not convert food efficiently, do not grow at optimal rates in aquaculture settings, and hence live under constant stress conditions. As hatcheries begin to introduce domesticated F1s into their breeding populations, identifying the best performers and using them to replace aging, wild broodstock are essential to the continued growth of the industry.

Many aquaculture species, including halibut, are amenable to selection due to their high levels of genetic variation and high fecundity (Gjedrem 2005). Several selection methods, such as mass selection and among-family selection (Hershberger et al. 1990; Basiao and Doyle 1999; Rezk et al. 2003), have been successfully applied in aquaculture. Recently, more elaborate selection methods have been proposed, including walk-back, index, and marker-assisted selection (MAS) methods (Lande and Thompson 1990; Chevassus et al. 2004; Sonesson 2005, 2007).

The Impact of Generation Time

Unlike many aquaculture species, which have generation times of about 3 years, a female Atlantic halibut is not ready for high-quality spawning for about 10 years. Thus, a relatively simple family selection scheme, whereby the top 10% of performers from each family are selected, raised to sexual maturity, and crossed with another high performing family, would take about 20 years for this species. Associated with this long time frame are the space and rearing costs of maintaining reasonable numbers of potential broodstock. Using standard selection approaches for halibut, broodstock enhancement is clearly a costly and risky proposition for this young industry.

Marker-Assisted Selection

With the construction of a genetic map and the ongoing QTL studies, genetic markers can be introduced into the selection regime for halibut. MAS is the selection method whereby the breeding value of a parent is estimated by including genotypic information from QTL markers for the trait of interest. A major advantage of MAS is that selection is based on genotype and thus selection can be done on juveniles, which avoids the costs of raising nonselected fish to the production stage. MAS is particularly useful for improving selection efficiency when heritability is low (<0.5) (Lynch and Walsh 1997; Dekkers and Hospital 2002). An additional advantage to MAS is the ability to select for traits that require potentially lethal measurements (i.e., disease resistance, flesh quality, fillet yield), since trait values are not determined on the selection candidates. In phenotypic selection, siblings of the best performers are selected, while MAS allows the selection of siblings with similar genotypes, which increases the selection efficiency. Approaches that consider both phenotypic and genotypic information in a selection index (Lande and Thompson 1990) are likely to provide further increases in selection efficiency.

Selection efforts in Atlantic halibut in Canada are well underway. Since 1999, 100–1500 fish from each year class have been tagged, their pedigree determined, and phenotypic and family performance data collected. This information will be developed into a selection index that will include growth QTL

data from the current experiment. The pedigree and phenotypic data collected since 1999 will be used to confirm and identify high growth QTL in these broodstock. The approach of collecting the phenotypic data prior to QTL identification is essential to compensate for the long generation time of halibut. This allows the selection index with QTL data to be applied almost immediately since the 1999 year class fish will soon be incorporated into the production broodstock.

FUTURE DIRECTIONS

Halibut aquaculture presents challenges that are unique among aquaculture species. The development of all-female halibut lines will have an immediate impact on production and the economic viability of the industry. Genetic and genomics tools have already substantially improved our understanding of the reproductive success and family structure of halibut production runs and will be key aspects of future broodstock selection programs. The identification of QTL and their incorporation into the selection index will further increase the ability to select high-performing broodstock. The investment in genetic and genomic research on halibut has made a significant contribution to the progress in this industry over the last ten years.

ACKNOWLEDGEMENTS

We acknowledge the efforts of our colleagues and partners who have contributed to the work: Brian Blanchard, Melissa Rommens, Nick Brown, David Raymond, Frank Powell, Shawn Raymond, Sid Raymond, Colin McGowan, Makoto Matsuoka, Tim Jackson, Chris Hendry, Harald Tvedt, Vikki Hughes, Stephanie Warrington, Cheryl Brooking, Joy Wade, Cheryl-Anne Smith, Sue Douglas, Leah Knickle, Colleen Murphy, and Harry Murray. We thank Sue Douglas for a critical review of the manuscript. This is NRCC publication 2009-42820.

REFERENCES

Baroiller JF and D'Cotta H. 2001. Environment and sex determination in farmed fish. Comp Biochem Physiol C. 130: 399–409.

Basiao ZU and Doyle RW. 1999. Test of size-specific mass selection for Nile tilapia, *Oreochromis niloticus*, cage farming in the Philippines. Aquacult Res. 30: 373–378.

Benfey TJ. 2009. Producing sterile and single-sex populations of fish for aquaculture. In: New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management (eds. Burnell G and Allan G). Woodhead Publishing Ltd, Cambridge, UK. pp 143–164.

Björnsson B. 1995. The growth pattern and sexual maturation of Atlantic halibut (*Hippoglossus hippoglossus* L.) reared in large tanks for 3 years. Aquaculture. 138: 281–290.

Brown N. 2002. Flatfish farming systems in the Atlantic region. Rev Fish Sci. 10: 403–419.

Brown NP, Bromage NR, Penman DJ, and Shields RJ. 1997. The karyotype of the Atlantic halibut, *Hippoglossus hippoglossus* (Linnaeus). Aquacult Res. 28: 489–491.

Chevassus B, Quillet E, Krieg F, Hollebecq MG, Mambrini M, Labbe L, Hiseux JP, and Vandeputte M. 2004. Enhanced individual selection for selecting fast growing fish: the "PROSPER" method, with application on brown trout (*Salmo trutta fario*). Genet Sel Evol. 36: 643–661.

Chistiakov DA, Hellemans B, and Volckaert FAM. 2006. Microsatellites and their genomic distribution, evolution, function and applications: a review with special reference to fish genetics. Aquaculture. 255: 1–29.

Coughlan J, Stefansson M, Galvin P, Dillane E, FitzGerald R, and Cross TF. 2000. Isolation and characterization of 11 microsatellite loci in Atlantic halibut (*Hippoglossus hippoglossus* L.). Mol Ecol. 9: 822–824.

Cross TF, Coughlan J, Burnell G, Cross MC, Dillane E, Stefansson MO, and Wilkins NP. 2005. Utility of microsatellite loci for detecting reduction of variation in reared aquaculture strains compared with wild progenitors and also as genetic "tags" in breeding programmes: evidence from abalone, halibut and salmon. Aquaculture. 247: 9–10.

Danzmann RG and Gharbi K. 2007. Linkage mapping in aquaculture species. In: Aquaculture Genome Technologies (ed. Liu ZJ). Blackwell Publishing, Ames, IA. pp 139–167.

Dekkers JCM and Hospital F. 2002. The use of molecular genetics in the improvement of agricultural populations. Nat Genet. 3: 22–32.

Devlin RH and Nagahama Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. 208: 191–364.

Douglas SE, Knickle LC, Kimball J, and Reith ME. 2007. Comprehensive EST analysis of Atlantic halibut (*Hippoglossus hippoglossus*), a commercially relevant aquaculture species. BMC Genomics. 8: 144.

Douglas SE, Knickle LC, Williams J, Flight RM, and Reith ME. 2008. A first generation Atlantic halibut *Hippoglossus hippoglossus* (L.) microarray: application to developmental studies. J Fish Biol. 72: 2391–2406.

Falconer DS and MacKay TFC. 1996. Introduction to Quantitative Genetics. Pearson Education Limited, Essex, UK.

Frank-Lawale AS. 2006. Genetic management of Atlantic halibut (*Hippoglossus hippoglossus*). PhD Thesis. Institute of Aquaculture, University of Stirling, Scotland, UK.

Gjedrem T (ed.). 2005. Selection and Breeding Programs in Aquaculture. Springer, Dordrecht, The Netherlands.

Godwin J, Luckenbach JA, and Borski RJ. 2003. Ecology meets endocrinology: environmental sex determination in fishes. Evol Dev. 5: 40–49.

Hedrick PW. 2007. Sex: differences in mutation, recombination, selection, gene flow, and genetic drift. Evolution. 61: 2750–2771.

Hendry CI, Martin-Robichaud DJ, and Benfey TJ. 2002. Gonadal sex differentiation in Atlantic halibut. J Fish Biol. 60: 1431–1442.

Hendry CI, Martin-Robichaud DJ, and Benfey TJ. 2003. Hormonal sex reversal of Altantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture. 219: 769–781.

Hershberger WK, Meyers JM, McAuley JM, and Saxton AM. 1990. Genetic changes in growth of coho salmon (*Onchrhynchus kisutch*) in marine netpens, produced by ten years of selection. Aquaculture. 85: 187–197.

Hughes V, Benfey TJ, and Martin-Robichaud DJ. 2008. Effect of rearing temperature on sex ratio in juvenile Atlantic halibut, *Hippoglossus hippoglossus*. Env Biol Fish. 81: 415–419.

Imsland AK and Jonassen TM. 2005. The relation between age at first maturity and growth in Atlantic halibut (*Hippoglossus hippoglossus*) reared at four different light regimes. Aquacult Res. 36: 1–7.

Jackson TR, Martin-Robichaud DJ, and Reith M. 2003. Application of DNA markers to the management of Atlantic halibut (*Hippoglossus hippoglossus*) broodstock. Aquaculture. 220: 245–259.

Jákupsstovu SH and Haug T. 1988. Growth, sexual maturation and spawning season of Atlantic halibut, *Hippoglossus hippoglossus*, in Faroese waters. Fish Res. 6: 201–215.

Kolbehdari D, Jansen GB, Schaeffer LR, and Allen BO. 2005. Power of QTL detection by either fixed or random models in half-sib designs. Genet Sel Evol. 37: 601–614.

Kondo M, Nagao E, Mitani H, and Shima A. 2001. Differences in recombination frequencies during female and male meiosis of the sex chromosomes of medaka, *Oryzias latipes*. Genet Res. 78: 23–30.

Korol A, Shirak A, Cnaani A, and Hallerman EM. 2007. Detection and analysis of quantitative trait loci (QTL) for economic traits in aquatic species. In: Aquaculture Genome Technologies (ed. Liu Z). Blackwell Publishing, Ames IA. pp. 169–197.

Lande R and Thompson R. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics. 124: 743–756.

Liu BH. 1998. Statistical Genomics: Linkage, Mapping, and QTL Analysis. CRC Press, New York.

Lynch M and Walsh B. 1997. Genetics and analysis of quantitative traits. Sinauer Associates Inc, Sunderland, MA.

Lynn A, Schrump S, Cherry J, Hassold T, and Hunt P. 2005. Sex, not genotype, determines recombination levels in mice. Am J Hum Genet. 77: 670–675.

MacKay TFC. 2001. The genetic architecture of quantitative traits. Annu Rev Genet. 35: 303–339.

Massault C, Bovenhuis H, Haley C, and de Koning D-J. 2008. QTL mapping designs for aquaculture. Aquaculture. 285: 23–29.

McGowan C and Reith ME. 1999. Polymorphic microsatellite markers for Atlantic halibut, *Hippoglossus hippoglossus*. Mol Ecol. 8: 1761–1763.

Nagahama Y. 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol Biochem. 31: 105–109.

Norberg B, Valkner V, Huse J, Karlsen I, and Grung GL. 1991. Ovulatory rhythms and egg viability in the Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture. 97: 365–371.

Olsen Y, Evjemo JO, and Olsen A. 1999. Status of the cultivation technology for production of Atlantic halibut (*Hippoglossus hippoglossus*) juveniles in Norway/Europe. Aquaculture. 176: 3–13.

Penman DJ and Piferrer F. 2008. Fish gonadogenesis. Part I: genetic and environmental mechanisms of sex determination. Rev Fish Sci. 16: 14–32.

Piferrer F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture. 197: 229–281.

Piferrer F, Zanuy S, Carrillo M, Solar II, Devlin RH, and Donaldson EM. 1994. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J Exp Zool. 270: 255–262.

Power JE. 2009. Cage culture characteristics of juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). M.Sc. Thesis. Department of Biology, University of New Brunswick.

Reid DP, Pongsomboon S, Jackson TR, McGowan C, Murphy C, Martin-Robichaud DJ, and Reith M. 2005. Microsatellite analysis indicates an absence of population structure among *Hippoglossus hippoglossus* in the north-west Atlantic. J Fish Biol. 67: 570–576.

Reid DP, Smith CA, Rommens M, Blanchard B, Martin-Robichaud D, and Reith M. 2007. A genetic linkage map of Atlantic halibut (*Hippoglossus hippoglossus* L.). Genetics. 177: 1193–1205.

Rezk MA, Smitherman RO, Williams JC, Nichols A, Kucuktas H, and Dunham RA. 2003. Response to three generations of selection for increased body weight in channel catfish, *Ictalurus punctatus*, grown in earthen ponds. Aquaculture. 2003: 69–79.

Sonesson AK. 2005. A combination of walk-back and optimum contribution selection in fish: a simulation study. Genet Sel Evol. 37: 587–599.

Sonesson AK. 2007. Within family marker-assisted selection for aquaculture species. Genet Sel Evol. 39: 301–317.

Tanksley SD. 1993. Mapping polygenes. Annu Rev Genet. 27: 205–233.

Tvedt HB, Benfey TJ, Martin-Robichaud DJ, McGowan C, and Reith M. 2006. Gynogenesis and sex determination in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture. 252: 573–583.

van Nes S and Andersen O. 2006. Temperature effects on sex determination and ontogenetic gene expression of the aromatases cyp19a and cyp19b, and the estrogen receptors esr1 and esr2 in Atlantic halibut (*Hippoglossus hippoglossus*). Mol Reprod Dev. 73: 1481–1490.

Chapter 4

Prospects and Pitfalls of Clonal Fishes in the Postgenomic Era

Barrie D. Robison and Gary H. Thorgaard

BACKGROUND

Clonal fishes are populations of genetically identical individuals that can be produced through natural phenomena, such as parthenogenetic reproduction (Vrijenhoek 1994), or through experimental manipulation. They are often highly inbred, sometimes to the point of complete homozygosity. However, heterozygous clonal lines can occur naturally and can also be produced through line crossing. The genetic uniformity of clonal fishes almost always refers to the nuclear genome.

There are numerous examples of clonal fishes (Komen and Thorgaard 2007). Species in which clonal lines have been produced include the rainbow trout, loaches, carp, tilapia, and zebrafish. The biological questions that drive the production of clonal lines in these species are diverse and include the analysis of quantitative genetics, cytogenetics, reproductive biology, physiology, and behavioral genetics. The utility and history of clonal fishes has been discussed in a recent review by Komen and Thorgaard (2007). In addition, the utility of salmonid clonal lines has been recently reviewed by Nichols (2009). In this chapter, we discuss the prospects for utilizing clonal lines to address fundamental research in aquaculture, considering both the advantages and drawbacks of this fascinating genetic system.

Clonal Fishes: Harnessing the Power of Inbred Lines

Clonal fishes are highly analogous to inbred lines that have been widely utilized in other groups of animals and plants (Zhou and Lamont 1999; Beck et al. 2000; Liu et al. 2003) to greatly facilitate genomics research. Inbred lines are classically produced by many generations of mating of relatives (e.g., brother $\times$ sister mating). The most salient features of the inbred line approach are the homozygosity and uniformity of the animals. This homozygosity simplifies genetic analyses by eliminating the effects of dominance and the complexity of multiple alleles at the loci under investigation. The homozygosity of inbred lines also creates significant advantages in longer-term research projects. In particular, homozygous inbred lines do not evolve over time, other than through the accumulation of mutation. This lack of genetic variation results in a repeatable experimental system, producing a genetic background that can be held constant and uniform over time and between laboratories. The availability of a constant and uniform genetic background

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

ultimately facilitates the synthesis of numerous experiments into a general mechanistic theory of the biological system under investigation.

The history of the field of genetics is replete with examples of inbred lines that have been used to answer fundamental questions in genetics, medicine, physiology, and evolutionary biology. The inbred line approach has been used to make amazing scientific advances in a variety of organisms. In mice, a large number of inbred lines are housed in the Jackson Laboratory (Bar Harbor, ME; Lyon and Searle 1989). These lines have well-described phenotypes, and genetic analyses have resulted in exciting discoveries in numerous fields, including behavior (Crawley et al. 1997), physiology, and development (Hoit et al. 2002). Inbred lines have also been created for some agricultural species, such as the chicken and corn. In plants, *Arabidopsis* researchers have gone so far as to create recombinant inbred lines (RILs) from crosses of inbred parental lines (Lister and Dean 1993). These RILs have been extensively characterized for numerous important phenotypes, and detailed genotypic data are also available (El-Lithy et al. 2004). The availability of "pre-genotyped" inbred lines greatly accelerates studies of the genetic basis of complex traits, including flowering time, yield, and growth. Similar approaches have also been widely used in RILs resulting from crosses of mouse inbred lines (Belknap 1998).

Research advances have become even more dramatic as the power of the inbred line approach has been combined with recent advances in genomic technology. Current genome sequencing efforts involve the sequencing of multiple recombinant genomes derived from crosses of inbred lines of mice, *Arabidopsis*, and *Drosophila*. These efforts are resulting in unprecedented insights into the genetics and evolution of complex traits. These insights are transforming diverse biological disciplines, such as evolutionary biology, physiology, and animal behavior.

Clonal lines in aquaculture species are constructed to capitalize on the same features of inbred lines described above. Like inbred lines, clonal lines are homozygous, which leads to repeatability within and among experiments. However, there are some important differences between clonal lines and inbred lines; the advantages and disadvantages of which are discussed below.

Advantages of Clonal Lines

Speed of Generation

One of the primary advantages of the clonal line approach over the more traditionally constructed inbred lines is the speed with which clonal lines can be generated. Clonal lines of fishes can be created in just two generations, whereas homozygosity is only asymptotically approached in other organisms through many generations of close inbreeding. In the first generation of clonal line production, an animal of interest is used to construct uniparental homozygous diploid ("doubled haploid") offspring. The approach used depends on the sex of the parent individual. Male parents are used in androgenesis, where the sperm fertilize eggs that have been irradiated to destroy the maternal genome (Purdom 1969). Female parents originate the process of gynogenesis, where their eggs are fertilized by irradiated sperm, in which the paternal genome has been destroyed (Chourrout and Quillet 1982). In either case, the developing embryos are treated with either heat or pressure to suppress the first cleavage division and restore the diploid condition. Since the chromosomes all derive from a duplicated haploid set, the resultant offspring are all homozygous, but genetically distinct (just like each gamete from an outbred individual is genetically distinct).

Once the doubled haploid offspring reach maturity, a second round of androgenesis or gynogenesis is performed, using the unirradiated gametes of the doubled haploid fish. Since these fish are homozygous, their gametes are all genetically identical. The second round of chromosome set manipulation then results in a population of fish that are homozygous and genetically identical: a clonal line.

This two-generation approach is much faster than the many generations of sib matings it would take to achieve even 90% homozygosity. The accelerated production of the clonal line approach has two advantages. The first, and most obvious, is the reduction in time, enabling research projects on a 3- to 6-year timescale. This is particularly advantageous with aquaculture species with long generation times. For example, many salmonid species have

generation times approaching 3–4 years. Creation of inbred lines using traditional sib mating would take 15–20 years, easily eclipsing the timescales associated with graduate student projects and normal funding cycles.

The second advantage of the accelerated production of clonal lines is less obvious. If one is interested in "fixing" an interesting genotype for subsequent experimentation, the genetics of a clonal line will not change during line development. In rainbow trout, this approach has been used to fix genotypes for a variety of interesting traits. For example, we have shown that the accelerated development rate exhibited by the Swanson clonal line of rainbow trout (Robison et al. 1999) derived from an Alaskan population is a reflection of that phenotype in the source population from which the clone was derived (Robison and Thorgaard 2004). This is important because it shows that interesting variations in phenotypes relevant to aquaculture can be captured in a stable and repeatable genetic system. Because of their homozygosity and the manner in which they are propagated, if the trait is found in the initial doubled haploid generation, the phenotype should be stable in subsequent generations of clones.

Storage Using Cryopreserved Sperm

Another advantage of the clonal line approach with salmonid fishes is the ease of storage of cryopreserved sperm. Established protocols exist, in which sperm can be frozen in liquid nitrogen and reconstituted years later (Tiersch and Mazik 2000; Chapter 20 of this book by Herráez et al.). Thus, any given male clonal line can be almost indefinitely maintained and resurrected after accidental loss. The line can also be archived using this approach if there is no sufficient space or funding to maintain live animals. Frozen sperm also facilitates the transfer of clonal lines across laboratories. Sperm samples can be shipped frozen around the world, reconstituted upon receipt, and live animals generated in the home laboratory. The live animals can be generated through androgenesis, creating a true clone of the nuclear genotype. This minimizes shipping mortality of newly hatched fry. This approach can even apply to female lines, as sex reversal of XX fish is possible using testosterone baths.

The cryopreservation of clonal line sperm is essential for line banking in fishes because the cryopreservation of live embryos, a technique necessary for inbred line maintenance in other vertebrate model species, has not yet been successful in fishes (Tiersch and Mazik 2000).

One disadvantage of the cryopreservation of clonal lines using frozen sperm is the fact that only the nuclear genotype is maintained in the line. The mitochondrial genome is still maternally inherited after androgenesis (May and Grewe 1993; Brown and Thorgaard 2002). Androgenesis creates homozygous clones of the donor sperm with the mitochondrial genetic background from the female that contributed the irradiated eggs. While the irradiated eggs appear to harbor no functional nuclear genetic material (unless chromosome fragments are transmitted), the female mitochondrial genotype is maintained. This has significant implications if the biological system one wishes to investigate is influenced by variation in mitochondrial function (Brown et al. 2006). Phenotypes such as swim performance, muscle and cardiac physiology, aging, and oxygen utilization are all influenced by mitochondrial genotype (Mannen et al. 1998; Wallace 1999). In addition, any phenotypes influenced by cytonuclear interactions would also be difficult to dissect using this approach.

Ease of Mitochondrial Transfer

Conversely, the fact that a consistent nuclear genotype can be "installed" upon different cytoplasmic backgrounds creates an interesting research opportunity. If one is interested in the role of mitochondrial genotype on phenotype, what better system than androgenetic clonal lines? In this case, genomic technologies can be used to genotype mitochondria of different females and study the effect of specific mitochondrial haplotypes on performance. Once again, the advantage of the clonal line system in this context is the speed with which experimental animals can be generated. In one generation, a researcher could create a series of lines identical in nuclear genotype but harboring known variants in mitochondrial genotype. Different mitochondrial genomes have been speculated to be functionally distinct (Mannen et al. 1998; Brown et al. 2006), but

definitive evidence addressing this question has been challenging to collect.

Disadvantages of Clonal Lines

Poor Survival Rates

While there are clear advantages to using clonal lines in aquaculture genomics research, the system is by no means perfect. One of the biggest disadvantages of this approach is the poor survival of the clones, which has two potential (and not mutually exclusive) causes (Thorgaard et al. 1990). The first cause is technical, in which the treatments used to reestablish diploidy—egg irradiation for androgenesis, followed by either a pressure or heat shock—cause mortality during development. Thus, the survival rates of clonal lines from fertilization to hatch are markedly lower than that of untreated controls.

However, adult clonal fish can also exhibit reduced survivorship. While lingering effects of egg irradiation or the treatments to restore diploidy discussed above may play a role in reducing survival, one must also consider quantitative genetics. Establishing homozygosity in a single generation should eliminate all lethal recessive alleles (assuming no epistatic masking effects), but may still induce a substantial reduction in fitness through fixation of sublethal deleterious alleles. In other words, inbreeding depression in homozygous clones is severe. For experiments requiring large numbers of animals, the inbreeding depression of fully homozygous lines must often be mitigated through the production of either hybrid clonal lines or crosses to outbred animals (see below).

Chromosome Fragments

A second disadvantage of clonal lines of fishes can be the persistence of chromosome fragments in some instances. Identifying the optimal radiation treatment for the gametes can be challenging. Too high a dose can result in nonfunctional gametes, while too low a dose can be associated with the presence of a large number of chromosome fragments that can impair embryo viability. Even with optimized treatments, some chromosome fragments may persist and be retained in the offspring (Thorgaard and Pearson 1985; Ocalewicz et al. 2004). In some instances, these fragments may even be passed on to subsequent generations (Peek et al. 1997). It appears that the presence of chromosome fragments is associated with reduced fertility in gynogenetic females (Krisfalusi et al. 2000). This phenomenon is still being studied in more detail, but it is important to keep in mind as a possible complication inherent to studies involving clonal fishes.

Poor Fertility of Females

A third challenge with clonal lines of fishes has been the relatively poor fertility of homozygous females. In our experience with rainbow trout, we have found that a high fraction of homozygous males that reach the age of sexual maturity produce functional sperm while a much lower fraction of homozygous females produce functional eggs (Paul Wheeler, personal communication). The relative contributions of inbreeding, treatment effects, and chromosome fragments to this problem have not been evaluated, but this is another factor to consider when establishing a research trajectory involving clonal lines.

Limited Sampling

A fourth limitation of clonal lines that must be acknowledged is that each line represents a very narrow sampling of the total genetic variation in a species. As an amplification of a single doubled haploid genome, a clonal line simply represents the genomic sampling of a single sperm or unfertilized egg. Clearly, it is important to recognize that results with a single clonal line cannot be generalized to a species as a whole.

How can we deal with this limitation? One solution could be to utilize a broad sampling of diverse clonal lines in research. Outbred strains require large numbers of individuals for successful propagation while avoiding inbreeding, and in the same amount of space, a very impressive sample of diverse clonal lines could be propagated. A second solution could be to develop clonal lines from diverse sources, characterize them, use them to identify quantitative trait loci (QTL) associated with traits distinct to the original source, and then test the source populations for the presence of the QTL found in the clonal lines. In effect, we can use the lines to allow us to develop testable hypotheses about the source populations.

CLONAL LINES: A REPEATABLE EXPERIMENTAL SYSTEM

One of the most profound advantages of the clonal line approach is the availability of a repeatable experimental system that facilitates long-term genetic research. However, the specifics of clonal line development offer three different types of experimental breeding designs, each with specific advantages and drawbacks.

Homozygous Clones

The first option is to use true homozygous clones, created through either androgenesis or gynogenesis. This approach creates a repeatable genetic system with no heterozygosity. However, the survivorship issues discussed earlier limit the application. Experiments that rely on large sample sizes are difficult to perform with this approach. Some of the most successful applications of the true homozygous clone approach include expression studies and whole genome sequencing (see below).

Hybrid Clones

A second experimental design is to create hybrid clones from two homozygous clonal lines. Here, we cross two established clonal lines to create a population of genetically identical fish that are also heterozygous. This approach requires no chromosome set manipulation, and the animals are genetically uniform but are not inbred. Because it is a normal cross between a homozygous female and a homozygous male and it does not involve androgenesis or gynogenesis, and because the progeny are not inbred, the survival of the embryos is greatly improved. One can therefore generate a significantly larger population of experimental animals. The repeatability of this system over the long term is not compromised, as genetically identical animals can be produced again and again, provided that the original clonal lines are still available to supply gametes. This consideration is easily achieved for male clonal lines, but less so for female clonal lines.

The hybrid clone experimental design is ideal for phenotypes in which variation is quantitative rather than qualitative (Bongers et al. 1998; Young et al. 1998).

Crosses of Clones to Outbred Animals

One way to circumvent the poor survival of truly clonal animals is to cross a clonal line of interest to outbred animals. This works best using clonal sperm, though it is technically possible with clonal eggs. Because the availability of eggs from outbred females will not be limiting, very large numbers of offspring can be studied with this approach.

With this approach, sperm from clonal lines with interesting phenotypes are used to fertilize the eggs of outbred females. One can use this approach to test for phenotypic variation among clonal lines. In this case, the eggs of the outbred female are subdivided in a factorial design, such that each clonal male fertilizes a subset of eggs from each female. Here, we are primarily interested in the genetic contribution of the sire, and variation in phenotypes among clonal lines can be detected by statistically testing for a sire effect.

The outbred cross approach will underestimate the true divergence among clonal lines, but we have successfully used it to demonstrate variation among lines in embryonic development rate (Robison et al. 1999). Since that time, the approach has also been used to identify genetic variation among clonal lines in other phenotypes, including behavior (Lucas et al. 2004) and disease resistance (Thorgaard et al., unpublished results). Once divergent clonal lines have been identified using this approach, detailed genetic analyses can be performed on the trait by crossing divergent clonal lines as described below.

GENETIC ANALYSES USING CLONE CROSSES

Genetic Mapping

Genetic maps are a central tool in genomics studies. They facilitate assembly of complete genome sequences, are central to the discovery of the genetic basis of quantitative traits, and can be used to study the evolution of genome structure.

The homozygosity of clonal lines greatly facilitates genetic mapping. Clonal rainbow trout have been used to create several genetic maps (Young et al. 1998; Nichols et al. 2003b; Lucas et al. 2004; Nichols et al. 2008) and have been consolidated with

maps derived from outbred populations (Nichols et al. 2003b).

Prior to the recent expansion of genomics technology, genetic maps were often constructed with dominant markers. Dominant markers (such as amplified fragment length polymorphisms (AFLPs)) do not display an observable difference between heterozygotes and homozygous dominant individuals. This is a considerable drawback when constructing genetic maps with outbred individuals. However, clonal lines can be used to create mapping panels of homozygous individuals (doubled haploids). These individuals unambiguously display their genotype, increasing the range of molecular markers available for mapping. The first use of this approach was in rainbow trout (Young et al. 1998), in which a panel of doubled haploid fish was used to create a genetic map using AFLP markers. Since then, this approach has also been expanded to QTL analyses (Robison et al. 2001).

Quantitative Trait Loci Studies

One of the most common and useful applications of clonal lines is their use in identifying QTL. QTL are regions of the genome that are statistically associated with a quantitative trait, presumably because these regions harbor one or more genes with allelic variants that affect the phenotype (called quantitative trait genes (QTG)). Identification of QTL, and ultimately QTG, is an important goal in aquaculture genetics because many of the phenotypes critical to the success of the industry are quantitative traits. Identification of QTL and QTG can ultimately lead to the application of marker-assisted selection in breeding programs. Quantitative traits are measured on a continuous scale and are influenced by both environmental and genetic variation. They are usually polygenic (influenced by multiple genetic loci), and the effects of an individual gene on the phenotype can range from small (<5% variance explained) to large (>25% variance explained).

QTL studies rely on the creation of genetic maps (described above), which can be facilitated by the use of homozygous clonal lines. Further, the homozygosity of clonal lines facilitates the identification of QTL using homozygous doubled haploid fish. In this system, recessive alleles that affect phenotype are unmasked, increasing the power to detect QTL (Martinez et al. 2005). In addition, dominant genetic markers can be used to identify QTL in a panel of doubled haploids. A disadvantage of this system is that it prevents the estimation of the degree of dominance for a given QTL.

UTILIZATION OF DNA OR RNA FROM CLONES

Expression Studies

Microarray studies often rely on small numbers of biological replicates. Genetic variation among normal outbred individuals can therefore be a major source of error variance in these studies. By using genetically uniform animals, experimental repeatability of such studies has been shown to be quite high. This has been demonstrated using inbred lines of *Drosophila* (Weber et al. 2008) and mice (Wei et al. 2004).

Clonal lines of fishes, with their ease and rapidity of generation, clearly have similar potential for utilization in gene expression studies. A few studies to date have confirmed this expectation. Purcell et al. (2006) were able to more clearly identify the genes that were expressed following virus infection by using clonal rainbow trout. Bayne et al. (2006) were able to identify differences in the liver and kidney transcriptomes among three clonal lines of rainbow trout. Xu et al. (2010) utilized advanced backcross lines in which a QTL associated with rapid embryonic development was introgressed into the background of a slower-developing line to identify a large number of genes associated with the rapid development phenotype. Clearly, there is considerable potential for broader utilization of clones in gene expression studies.

Identification of Duplicate Genes

Fishes in general have a high incidence of duplicated genes (Amores et al. 1998) and the salmonids in particular have an especially high incidence due to their tetraploid ancestry (Allendorf and Thorgaard 1984). A recent study (Sanchez et al. 2009) demonstrated that duplicate genes can be readily detected in rainbow trout using DNA from clonal sources. The presence of multiple forms of a gene in DNA from a homozygous clonal line represents evidence that the gene is duplicated. Without using DNA from

a homozygous source, identification of resolution of gene duplicates from allelic types would be problematic. The ability to identify duplicated genes using clonal lines highlights a very important contribution of clonal lines to aquaculture genomics: sequencing of whole genomes of cultured fishes.

Whole Genome Sequencing

Sequencing of whole genomes has increasingly become the norm in genetics research. Unfortunately, sequencing of genomes for aquaculture organisms has lagged behind other animals in agriculture and other research models. There are likely two reasons for this. First, biomedical research has a substantially higher total research funding than does aquaculture. Second, and more relevant, it represents the logistical difficulties associated with genome sequencing of aquaculture species. Aquaculture species (especially the salmonids) have large genomes (up to approximately 80% the size of the human genome) and some groups such as salmonids have tetraploid ancestries, with an associated high incidence of duplicate genes (Allendorf and Thorgaard 1984). Genome sequencing is a complex process that requires assembly of genome fragments, and the size and complexity of genomes of aquaculture species make assembly of their genomes extremely difficult. Even with the availability of high-throughput sequencing methods, the genomes of aquaculture species will be challenging to sequence (Quinn et al. 2008), and doubled haploid material appears to provide the best prospect for unambiguous assembly (Waldbieser et al. 2010).

Fortunately, clonal lines can facilitate genome sequencing for aquaculture species. Clonal lines are homozygous, which means that there is (almost) no allelic variation within an individual. Sequence variation in closely related genes is therefore usually a result of gene duplication. Thus, assembly of duplicated gene regions is facilitated by application of homozygous clones. The current Atlantic salmon genome sequencing project uses a doubled haploid Atlantic salmon (Unni Grimmholt, personal communication). Similarly, the rainbow trout genome project in France is utilizing the Swanson doubled haploid clonal line (Yann Guigen, INRA, France, personal communication).

CASE EXAMPLES OF POTENTIAL FOR UTILIZING CLONES IN AQUACULTURE-RELATED RESEARCH

Despite their many advantages, clonal lines of trout have yet to be used to attack many of the most pressing problems in modern salmonid aquaculture. In this section, we outline several potential experimental paradigms in which clonal lines could be used as a tool to address a fundamental knowledge gap in aquaculture research. Where possible, we highlight advantages difficult to realize with more traditional approaches. We also present potential pitfalls inherent to the clonal line system.

Alternative Feeds in Aquaculture

Current aquaculture diets fed to salmonids rely on fish meal as a protein source. This protein source is critical, as salmonids do not appear to adequately mobilize carbohydrates as an energy source. The use of fish meal as a protein source in aquaculture diets is also problematic because of declining stocks of fish worldwide. This decline, coming at a time of increased demand for aquaculture products, precipitates an increase in cost. Ultimately, increased cost of fish meal increases the cost of production, threatening the viability of the industry.

In addition to the financial problems caused by declines in fish meal supply, there is a very real issue in terms of sustainability. Depletion of ocean sources of fish meal is a major conservation problem worldwide. Currently, most marine forage fisheries are either fully exploited or over exploited (Alder et al. 2008). As aquaculture comprises an ever-growing demand for these products (along with swine and poultry), continued sustainability of the aquaculture industry depends on a qualitative shift away from dependence on forage fisheries for fish meal and fish oil.

One of the strategies being investigated that may ameliorate the fish meal problem involves substitution of alternative protein sources into fish diets (Naylor et al. 2009). These protein sources are varied and include both animal protein and plant protein. However, the physiological response to alternative diet formulations is poorly understood, a knowledge gap that hinders implementation of these measures. Further, implementation of alternative diets may

require creation of new strains of fish that are better adapted to non-fish-meal-based diets.

What are the physiological factors that most directly limit the use of alternative protein sources? In order to establish a detailed understanding of the physiological response to specific nutrient manipulations, we can capitalize on the repeatability of clonal trout. By using clonal lines, we can establish a research trajectory in which the genetic background is held constant, both across experiments and over time. This can be very valuable, especially when studying physiological systems in which subtle changes have large effects.

In this scenario, substitution of alternative protein for fish meal can be studied in detail, using either pure clones or hybrid clones as appropriate. Animals can be subdivided into groups devoted to control and treatment diets, and their physiology studied in detail. Tests of hypotheses, and subsequent refinements of these hypotheses, can be conducted over time on the exact same genetic background, eliminating potentially troublesome genetic variation that may confound comparisons across experiments. This approach can be particularly advantageous when experiments involve more than one laboratory, as is often the case with these types of complex and important research questions. In this case, variation among results can be directly attributed to variation in the laboratory environment, rather than among strains of fish.

While the constancy of genetic background is an obvious plus for physiological experiments, the use of clonal lines in this situation is not without potential difficulties. In this case, the potentially low numbers of animals may limit larger experiments. However, detailed physiological studies do not usually employ the large sample sizes of typical genetic experiments. Experiments with sample sizes of 6–12 per treatment group are not uncommon in physiological studies, primarily because the measurements taken are very time-consuming. These experiments are easily accommodated by the clonal line paradigm. Larger experimental designs must be considered carefully, lest they be doomed by small sample size and therefore reduced statistical power.

One further consideration of clonal lines in this context is the potential lack of generality in the results. The constancy of the genetic system is an advantage, but in order for the research to translate to the industry, research using clonal lines must discover general phenomena that apply to industry fish. If the physiological response to an alternative diet is restricted to a particular genotype, the utility of the research is limited. One should therefore consider using a panel of clonal genotypes in the broader experimental approach. This approach capitalizes on the advantages of the clonal line system, while accounting for genetic variation in physiological responses. Alternatively, development of clonal lines directly from the aquaculture stocks that are in production may help mitigate this problem.

The example that we have cited for studying performance with alternative diets might be generalized to the use of clonal lines as controls in other performance trials. Quantitative genetic selection experiments normally include simultaneous propagation of unselected control lines in order to account for possible changes in culture conditions, which otherwise might not be detected. Inclusion of such controls can be costly in both space and time. The utilization of clonal fishes that maintain consistency of performance over space and time should be considered to meet such needs. Hybrid clones in some instances may show performances similar to those of normal outbred fishes (Muller-Belecke and Horstgen-Schwark 2000), and in such cases, inclusion of hybrid clones or panels of hybrid clones could represent a viable alternative as controls in selection experiments.

In addition to the quickly generated repeatable genetic background described here, clonal lines could be used to create inbred lines of fish from strains selected for increased performance when fed alternative diets. This approach, in which genetic variation is "captured" in a clonal line from its population of origin, will be described in the next section.

Temperature Tolerance

Temperature tolerance is critical to salmonid aquaculture, and inadequate temperature regulation in aquaculture settings can have disastrous consequences. In aquaculture, it can be advantageous to breed strains that have broader or increased temperature tolerance. To do this efficiently, we need to know the genetic basis of this trait. While there has been some work in salmonids, examining the

genetic basis of thermal tolerance (Somorjai et al. 2003), we still know surprisingly little about the specific genes and polymorphisms underlying identified QTL. The excellent performance of Arctic charr at low temperatures suggests that variation may exist among northern and high-altitude populations of some species that could be advantageous for cold water aquaculture.

There is considerable variation in tolerance for high temperature among wild populations of rainbow trout. In particular, "redband" varieties are thought to be remarkably tolerant to high temperatures (Thorgaard 2007). Incorporating these thermal tolerance alleles into cultured strains, without sacrificing increased growth, would be desirable. Clonal lines can be used to capture genetic variation that exists in wild strains (Robison and Thorgaard 2004). The clonal lines derived from thermally tolerant source populations would carry alleles at thermal tolerance QTL that could be bred into cultured populations through marker-assisted selection. This approach is reminiscent of selected lines in other agriculture species, except that the selection has occurred in the natural habitat.

To capture thermal tolerance alleles, redband trout could be sampled from systems known for large temperature fluctuations. Ideally, the ecological data on thermal maxima would be corroborated with laboratory-based experiments establishing true physiological superiority with regard to thermal preference (Rodnick et al. 2004). In some cases, fish may have apparent thermal tolerance, which is accomplished by behavioral mitigation strategies, such as seeking out cooler areas of a stream. Cultured species exposed to thermal stress have no such opportunities, and we are therefore interested in strains that have true physiological adaptations to thermal stress.

Once thermally tolerant fish are identified, their gametes can be used to create clonal lines through either androgenesis or gynogenesis. Resulting progeny of this generation can be tested in thermal preference and tolerance assays, and the animals with the highest tolerance selected for a subsequent round of androgenesis or gynogenesis. This step has the added advantage of selection in a homozygous state, potentially fixing recessive alleles that increase performance.

Once the second round of uniparental propagation is complete, we are left with clonal lines of fish harboring alleles for increased thermal preference. At this stage, the animals can be used in traditional QTL studies to identify the genomic regions harboring desirable alleles. In addition, we can use these studies to estimate the additive effect on each QTL. If alleles of large effect (explaining more than 25% of the phenotypic variance) are identified, these can be the focus of more intensive gene discovery studies or implemented in marker-assisted selection strategies.

This approach represents a long-term research agenda. To date, the relative paucity of genomics resources in rainbow trout and other aquaculture species has limited identification of the genetic variants underlying QTL. With the advent of next-generation sequencing technologies and genome projects for both Atlantic salmon and rainbow trout, progress toward this goal should accelerate considerably.

Domestication

The process of domestication, or adaptation of wild animals to captivity (Price 2002), is fundamental to aquaculture. Unlike many other agriculture species, most cultured fishes still have extant populations of the same species in their natural habitats. The domestication of aquaculture species is therefore very recent and ongoing. In fact, with some new aquaculture species, such as Atlantic cod and striped bass, cultured stocks are genetically indistinguishable from wild populations.

The adaptations involved in domestication are a result of the drastically different fitness landscape in the culture environment relative to the wild (Price 2002). Hallmarks of the culture environment include a complete lack of predation pressure, increased densities, increased availability of feed, reduced habitat complexity, and human intervention during reproduction. These differences impose significant selection pressure and result in phenotypic changes in behavior (e.g., increased "tameness"), physiology (e.g., attenuation of the stress response), disease resistance, and reproduction.

In addition to the implicit factors that result in adaptation to captivity, aquaculture often induces evolutionary change through explicit application of

artificial selection. Strains are routinely selected for increased growth rate and increased disease resistance. Phenotypic change resulting from explicit selection (such as increased growth rate) is often associated with changes in other physiological systems (such as behavior and reproduction). These patterns of correlated evolution, typically caused by underlying genetic correlations, arise either from pleiotropic effects of genes on multiple traits or from linkage disequilibrium (Lynch and Walsh 1998).

The combination of implicit and explicit factors results in evolution during domestication of a common suite of behavioral and physiological phenotypes that are more amenable to the aquaculture environment. Unfortunately, we have little understanding of the genetic changes that drive this process. Knowledge of these genetic changes would help the aquaculture industry for a number of reasons. First, we can identify whether there are common molecular patterns that occur during domestication. Second, we can identify whether further improvement in a desirable trait (such as growth rate) is opposed by antagonistic pleiotropic effects (such as reduced reproduction or reduced swim speed; Reinbold et al. 2009). Third, identifying the molecular changes associated with domestication helps us understand the precise basis of trait improvements. For example, while we might be selecting for increased growth, the observed phenotypic increase may be a result in alleles that affect feed conversion or drive changes in behavior that increase feed consumption.

Clonal lines can be applied to study the genetic basis of domestication. Currently, clonal lines from both domesticated (OSU, Arlee, Hot Creek) and wild or semi-wild (Swanson, Whale Rock, Clearwater) strains of rainbow trout exist at Washington State University. These lines have "captured" phenotypic variation inherent to their respective strains of origin. Using a QTL approach, we can examine the genetic basis for variation in growth (Drew et al. 2007), disease resistance (Nichols et al. 2003a), immune response traits (Zimmerman et al. 2004), behavior (Lucas et al. 2004), and life history traits (Robison et al. 2001; Nichols et al. 2007; Nichols et al. 2008). As outlined previously, these identified QTL can then be narrowed to identify the causative polymorphisms. The rate at which this proceeds is likely to be accelerated as new sequencing and genotyping technologies are adopted.

The strength of the clonal line approach in the context of studying domestication lies in their repeatability. The lines represent a constant genetic system, which does not change over time (e.g., become increasingly domesticated) or across labs. This decreases confounding variation, an important point in the study of domestication. The phenotypes associated with domestication are very complex and often integrate multiple physiological systems (e.g., growth, behavior). In addition, the homozygosity of the clonal line system increases the power of QTL studies.

Sexual Dimorphism

There is a growing appreciation that many traits that are highly relevant to the aquaculture industry are sexually dimorphic in some fish species. These traits include performance traits (Battiprolu and Rodnick 2004; Farrar and Rodnick 2004) and behavior (Oswald and Robison 2008). In addition, there is evidence from the zebrafish that response to dietary manipulation, a key feature in the study of alternative protein sources in aquaculture diets, may vary between males and females (Robison et al. 2008). The genetic basis and physiological basis of these sex differences is unknown. Clonal lines of salmonids by their nature are monosex, consisting of either all XX (female) or all male (YY) individuals. However, XX clonal lines can be sex reversed using hormone treatments (Bye and Lincoln 1986) into genetically female but phenotypically male fish. This presents an opportunity to study the physiological basis of sexually dimorphic traits in a constant genetic background.

For example, experiments could be conducted, in which genetically identical fish were subjected to swim performance and cardiac performance trials. If the animals were still dimorphic under these conditions, it would suggest that the physiological context of being male or female (presumably driven by steroid hormones) results in dimorphism. Alternatively, if known dimorphic traits diminish or disappear under this scenario, it would suggest that alleles present on the X or Y, apart from sex determination loci, regulate that trait.

The production of backcross lines, in which the genetic background of one line is substituted into that of the other, could be another solution to the problem of studying sexually dimorphic traits in clonal fishes. For example, repeated backcrosses of a male clone into the background of a female clone could eventually produce an inbred line containing both males (XY) and females (XX). This would negate the time advantage of rapid generation that clonal fishes have, but could facilitate other studies such as those involving sexually dimorphic traits. Xu et al. (2010) were recently able to utilize backcross lines to address the nature of gene expression differences among two lines differing in development rate.

CONCLUSION

Clonal fishes are genetically identical populations that harbor many potential opportunities for aquaculture research. The advantages of the clonal line system are that they are a repeatable and homozygous genetic system that is quickly generated and easily archived using cryopreservation. The disadvantages of the system include poor survival, inbreeding, and the potential for chromosomal fragments. Clonal lines can be used to attack several fundamental issues in aquaculture research, including identifying the genetic basis of desirable phenotypes, the physiological response to alternative diets, and the causes and consequences of sexual dimorphism. The advent of new genomics technologies offers a great deal of synergy with the clonal line experimental paradigm and should greatly accelerate discoveries in aquaculture research.

REFERENCES

Alder J, Campbell B, Karpouzi V, Kaschner K, and Pauly D. 2008. Forage fish: from ecosystems to markets. Annu Rev Environ Resour. 33: 153–166.

Allendorf FW and Thorgaard G. 1984. Tetraploidy and the evolution of salmonid fishes. In: The Evolutionary Genetics of Fishes (ed. Turner BJ). Plenum Press, New York. pp 1–53.

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, and Postlethwait JH. 1998. Zebrafish hox clusters and vertebrate genome evolution. Science. 282: 1711–1714.

Battiprolu PK and Rodnick KJ. 2004. Gender and maturity differences in cardiac performance under glucose and glucose-free conditions in rainbow trout (*Oncorhynchus mykiss*). ICB. 44: 675.

Bayne CJ, Gerwick L, Wheeler PA, and Thorgaard GH. 2006. Transcriptome profiles of livers and kidneys from three rainbow trout (*Oncorhynchus mykiss*) clonal lines distinguish stocks from three allopatric populations. Comp Biochem Physiol D Genomics Proteomics. 1: 396–403.

Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, Festing MFW, and Fisher EMC. 2000. Genealogies of mouse inbred strains. Nat Genet. 24: 23–25.

Belknap JK. 1998. Effect of within-strain sample size on qtl detection and mapping using recombinant inbred mouse strains. Behav Genet. 28: 29–38.

Bongers ABJ, Sukkel M, Gort G, Komen J, and Richter CJJ. 1998. Development and use of genetically uniform strains of common carp in experimental animal research. Lab Anim. 32: 349–363.

Brown KH and Thorgaard GH. 2002. Mitochondrial and nuclear inheritance in an androgenetic line of rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 204: 323–335.

Brown KH, Lee RW, and Thorgaard GH. 2006. Use of androgenesis for estimating maternal and mitochondrial genome effects on development and oxygen consumption in rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol B Biochem Mol Biol. 143: 415–421.

Bye VJ and Lincoln RF. 1986. Commercial methods for the control of sexual-maturation in rainbow-trout (*Salmo-gairdneri* r). Aquaculture. 57: 299–309.

Chourrout D and Quillet E. 1982. Induced gynogenesis in the rainbow-trout—sex and survival of progenies production of all-triploid populations. Theor Appl Genet. 63: 201–205.

Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, Hitzemann RJ, Maxson SC, Miner LL, Silva AJ, Wehner JM, Wynshawboris A, and Paylor R. 1997. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology. 132: 107–124.

Drew RE, Schwabl H, Wheeler PA, and Thorgaard GH. 2007. Detection of qtl influencing cortisol levels in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 272: S183–S194.

El-Lithy ME, Clerkx EJM, Ruys GJ, Koornneef M, and Vreugdenhil D. 2004. Quantitative trait locus

analysis of growth-related traits in a new arabidopsis recombinant. Plant Physiol. 135: 444–458.

Farrar RS and Rodnick KJ. 2004. Sex-dependent effects of gonadal steroids and cortisol on cardiac contractility in rainbow trout. J Exp Biol. 207: 2083–2093.

Hoit BD, Kiatchoosakun S, Restivo J, Kirkpatrick D, Olszens K, Shao HF, Pao YH, and Nadeau JH. 2002. Naturally occurring variation in cardiovascular traits among inbred mouse strains. Genomics. 79: 679–685.

Komen H and Thorgaard GH. 2007. Androgenesis, gynogenesis and the production of clones in fishes: a review. Aquaculture. 269: 150–173.

Krisfalusi M, Wheeler PA, Thorgaard GH, and Cloud JG. 2000. Gonadal morphology of female diploid gynogenetic and triploid rainbow trout. J Exp Zool. 286: 505–512.

Lister C and Dean C. 1993. Recombinant inbred lines for mapping rflp and phenotypic markers in *Arabidopsis-thaliana*. Plant J. 4: 745–750.

Liu KJ, Goodman M, Muse S, Smith JS, Buckler E, and Doebley J. 2003. Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics. 165: 2117–2128.

Lucas MD, Drew RE, Wheeler PA, Verrell PA, and Thorgaard GH. 2004. Behavioral differences among rainbow trout clonal lines. Behav Genet. 34: 355–365.

Lynch M and Walsh B. 1998. Genetics and Analysis of Quantitative Traits. Sinauer, Sunderland, MA.

Lyon MF and Searle AG (eds.). 1989. Genetic variants and strains of the laboratory mouse. Oxford University Press, Oxford.

Mannen H, Kojima T, Oyama K, Mukai F, Ishida T, and Tsuji S. 1998. Effect of mitochondrial DNA variation on carcass traits of Japanese Black cattle. J Anim Sci. 76: 36–41.

Martinez V, Thorgaard G, Robison B, and Sillanpaa MJ. 2005. An application of Bayesian QTL mapping to early development in double haploid lines of rainbow trout including environmental effects. Genet Res. 86: 209–221.

May B and Grewe PM. 1993. Fate of maternal mtDNA following co-60 inactivation of maternal nuclear-DNA in unfertilized salmonid eggs. Genome. 36: 725–730.

Muller-Belecke A and Horstgen-Schwark G. 2000. Performance testing of clonal oreochromis niloticus lines. Aquaculture. 184: 67–76.

Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Forster I, Gatlin DM, Goldburg RJ, Hua K, and Nichols PD. 2009. Feeding aquaculture in an era of finite resources. Proc Nat Acad Sci USA. 106: 15103–15110.

Nichols KM. 2009. Clonal lines and chromosome manipulation for aquaculture research and production. In: Molecular Research in Aquaculture. (ed. Overturf K). Wiley-Blackwell, Ames, IA. pp 195–216.

Nichols KM, Bartholomew J, and Thorgaard GH. 2003a. Mapping multiple genetic loci associated with *Ceratomyxa shasta* resistance in *Oncorhynchus mykiss*. Dis Aquat Org. 56: 145–154.

Nichols KM, Broman KW, Sundin K, Young JM, Wheeler PA, and Thorgaard GH. 2007. Quantitative trait loci x maternal cytoplasmic environment interaction for development rate in *Oncorhynchus mykiss*. Genetics. 175: 335–347.

Nichols KM, Edo AF, Wheeler PA, and Thorgaard GH. 2008. The genetic basis of smoltification-related traits in *Oncorhynchus mykiss*. Genetics. 179: 1559–1575.

Nichols KM, Young WP, Danzmann RG, Robison BD, Rexroad C, Noakes M, Phillips RB, Bentzen P, Spies I, Knudsen K, Allendorf FW, Cunningham BM, Brunelli J, Zhang H, Ristow S, Drew R, Brown KH, Wheeler PA, and Thorgaard GH. 2003b. A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). Anim Genet. 34: 102–115.

Oswald M and Robison BD. 2008. Strain-specific alteration of zebrafish feeding behavior in response to aversive stimuli. Can J Zool-Rev Can Zool. 86: 1085–1094.

Ocalewicz K, Babiak I, Dobosz S, Nowaczyk J, and Goryczko K. 2004. The stability of telomereless chromosome fragments in adult androgenetic rainbow trout. J Exp Biol. 207: 2229–2236.

Peek AS, Wheeler PA, Ostberg CO, and Thorgaard GH. 1997. A minichromosome carrying a pigmentation gene and brook trout DNA sequences in transgenic rainbow trout. Genome. 40: 594–599.

Price EO. 2002. *Animal Domestication and Behavior*. CABI publishing, New York. 297pp.

Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, and Park LK. 2006. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol. 43: 2089–2106.

Purdom CE. 1969. Radiation-induced gynogenesis and androgenesis in fish. Heredity. 24: 431–444.

Quinn NL, Levenkova N, Chow W, Bouffard P, Boroevich KA, Knight JR, Jarvie TP, Lubieniecki KP, Desany BA, Koop BF, Harkins TT, and Davidson WS. 2008. Assessing the feasibility of gs flx

pyrosequencing for sequencing the atlantic salmon genome. BMC Genomics. 9: 404.

Reinbold D, Thorgaard GH, and Carter PA. 2009. Reduced swimming performance and increased growth in domesticated rainbow trout, *Oncorhynchus mykiss*. Can J Fish Aquat Sci. 66: 1025–1032.

Robison BD and Thorgaard GH. 2004. The phenotypic relationship of a clonal line to its population of origin: rapid embryonic development in an Alaskan population of rainbow trout. Trans Am Fish Soc. 133: 455–461.

Robison BD, Drew RE, Murdoch GK, Powell M, Rodnick KJ, Settles M, Stone D, Churchill E, Hill RA, Papasani MR, Lewis SS, and Hardy RW. 2008. Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*). Comp Biochem Physiol D Genomics Proteomics. 3: 141–154.

Robison BD, Wheeler PA, Sundin K, Sikka P, and Thorgaard GH. 2001. Composite interval mapping reveals a major locus influencing embryonic development rate in rainbow trout (*Oncorhynchus mykiss*). J Hered. 92: 16–22.

Robison BD, Wheeler PA, and Thorgaard GH. 1999. Variation in development rate among clonal lines of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 173: 131–141.

Rodnick KJ, Gamperl AK, Lizars KR, Bennett MT, Rausch RN, and Keeley ER. 2004. Thermal tolerance and metabolic physiology among redband trout populations in south-eastern Oregon. J Fish Biol. 64: 310–335.

Sanchez CC, Smith TPL, Wiedmann RT, Vallejo RL, Salem M, Yao JB, and Rexroad CE. 2009. Single nucleotide polymorphism discovery in rainbow trout by deep sequencing of a reduced representation library. BMC Genomics. 10: 559.

Somorjai IML, Danzmann RG, and Ferguson MM. 2003. Distribution of temperature tolerance quantitative trait loci in Arctic charr (*Salvelinus alpinus*) and inferred homologies in rainbow trout (*Oncorhynchus mykiss*). Genetics. 165: 1443–1456.

Thorgaard GH and Pearson GD. 1985. DNA homologies between the rainbow-trout, chum salmon and coho salmon. Comp Biochem Physiol B Biochem Mol Biol. 81: 81–85.

Thorgaard GH, Robison BD, Wheeler PA, and Young WP. 2007. Possible approaches for genetic analysis of temperature adaptations in redband trout. In: Redband Trout: Resilience and Challenge in a Changing Landscape (eds. Schroeder RK and Hall JD). Oregon Chapter, American Fisheries Society, Corvallis. pp 19–24.

Thorgaard GH, Scheerer PD, Hershberger WK, and Myers JM. 1990. Androgenetic rainbow-trout produced using sperm from tetraploid males show improved survival. Aquaculture. 85: 215–221.

Tiersch TR and Mazik PM (eds.). 2000. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisianna.

Vrijenhoek RC. 1994. Unisexual fish—model systems for studying ecology and evolution. Annu Rev Ecol Syst. 25: 71–96.

Waldbieser GC, Bosworth BG, and Quiniou SMA. 2010. Production of viable homozygous, doubled haploid channel catfish (*Ictalurus punctatus*). Mar Biotechnol. 12 (4): 380–385.

Wallace DC. 1999. Mitochondrial diseases in man and mouse. Science. 283: 1482–1488.

Weber KE, Greenspan RJ, Chicoine DR, Fiorentino K, Thomas MH, and Knight TL. 2008. Microarray analysis of replicate populations selected against a wing-shape correlation in drosophila melanogaster. Genetics. 178: 1093–1108.

Wei CM, Li JN, and Bumgarner RE. 2004. Sample size for detecting differentially expressed genes in microarray experiments. BMC Genomics. 5: 87. Doi: 10.1186/1471-2164-5-87.

Xu P, Mcintyre LM, Scardina J, Wheeler PA, Thorgaard GH, and Nichols KM. 2010. Transcriptome profiling of embryonic development rate in rainbow trout advanced backcross introgression lines. Mar Biotechnol. 13 (4): 215–231. Doi:10.1007/s10126-010-9283-1

Young WP, Wheeler PA, Coryell VH, Keim P, and Thorgaard GH. 1998. A detailed linkage map of rainbow trout produced using doubled haploids. Genetics. 148: 839–850.

Zhou H and Lamont SJ. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim Genet. 30: 256–264.

Zimmerman AM, Evenhuis JP, Thorgaard GH, and Ristow SS. 2004. A single major chromosomal region controls natural killer cell-like activity in rainbow trout. Immunogenetics. 55: 825–835.

Part 2
Molecular Cytogenetics

Chapter 5

Application of Fluorescence In Situ Hybridization (FISH) to Aquaculture-Related Research

Ruth B. Phillips

INTRODUCTION

The technique of fluorescence in situ hybridization (FISH) has been used in localization of specific repetitive sequences, genetic markers, protein-coding genes, and transgenes to fish and shellfish chromosomes. These markers have been used to identify and characterize sex chromosomes, analyze interspecific hybrids, verify the chromosomal composition of chromosome-set manipulated finfish, and assign linkage groups to specific chromosomes in several species. In addition, FISH with species-specific probes is being used to localize pathogens in cultured shellfish and fish and document the composition and spatial location of microbial communities in aquaculture systems. Future applications include analyses of meiotic chromosomes and fine structure mapping of genes in regions of low recombination where large tracks of repetitive DNAs are present. Previous reviews that covered some of these topics include Phillips and Reed (1996), Phillips (2001), and Phillips (2007).

LOCALIZATION OF REPETITIVE SEQUENCES, TRANSPOSONS, AND TRANSGENES

Localization of repetitive sequences was the first application of FISH to fish and shellfish (reviewed in Phillips and Reed 1996; Phillips 2001; Martins 2007). Repetitive sequences include dispersed repeats that are often associated with transposons, tandem repeats that are usually found at centromeres or telomeres, and ribosomal DNA (rDNA). rDNA markers have been used in identification and management of several cultured fish species (reviewed by Foresti 2000). For example, rainbow trout (*Oncorhynchus mykiss*) currently cultured at the Experimental Center of Salmon Culture at Campos De Jordao, Brazil, have the rDNA on the long arms of chromosome 20, rather than the normal location on the short arms of this chromosome as shown by silver staining and FISH. Apparently, the fish stock originally imported from California, USA, had this variant karyotype, so this is a marker for descendants

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

of this introduced stock (Porto-Foresti et al. 2007). Closely related species often have the major (18S/5.8S/28S) rDNA locus at different chromosomal sites and intraspecific variation in the location, and amounts of the major rDNA locus is also fairly common. Thus, particular aquaculture strains may be identified by the location of major rDNA locus in the karyotype. Often, the multiple copies of 18S/5.8S/28S of the major rDNA cistron are located on a different chromosome pair than the minor cistron containing the 5S rDNA. Many fish species have two locations for the 5S rDNA, one of which may be on the sex chromosome pair (reviewed in Martins and Galetti 2001).

In rainbow trout, the 5S rDNA is found at two locations, one on chromosome 20 adjacent to the major rDNA cistron and the other on the short arm of the X chromosome (Phillips et al. 2004; Fig. 5.1A).

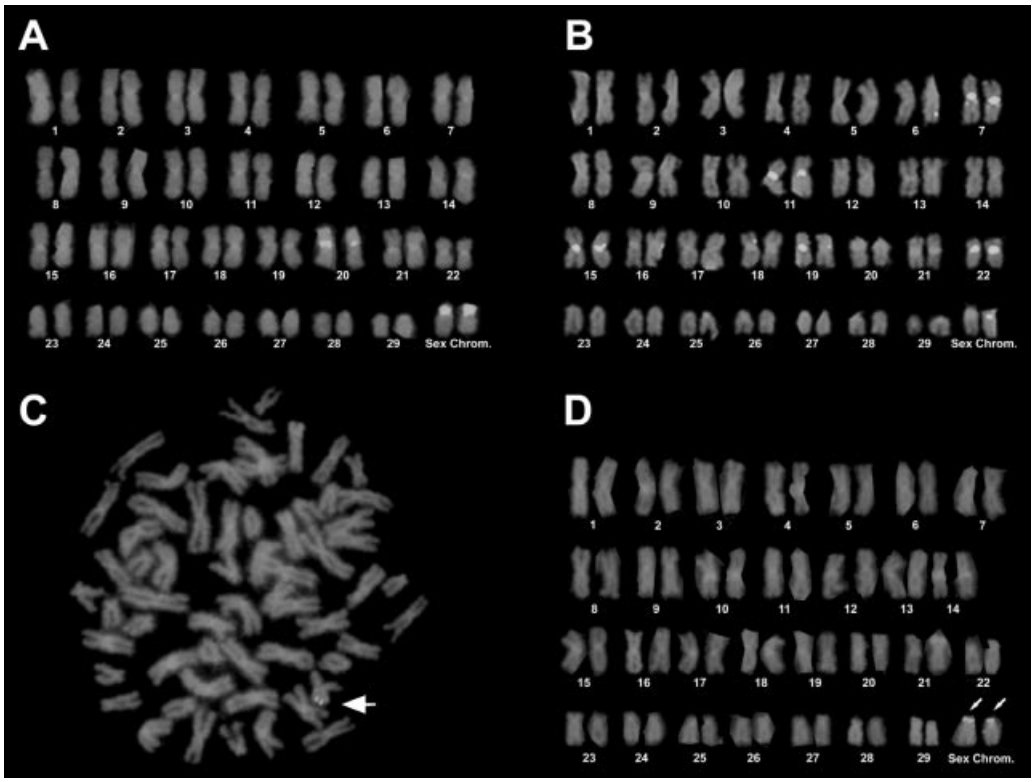


Figure 5.1. (**A**) Localization of 5S rRNA genes (labeled in red) in the rainbow trout karyotype. Note that there are two chromosomal pairs (20 and sex) with 5S rDNA with the largest amount of signal on the short arms of the X chromosomes (last chromosome pair in bottom row).
(**B**) Localization of the ID1B gene to rainbow trout chromosome Omy7 (last chromosome pair in top row) using a BAC probe containing ID1B (labeled in red) and the 10H19 centromere probe (labeled in green), which is specific to several chromosome pairs including Omy7.
(**C**) Localization of the growth hormone construct OnMTGH1 (labeled in red (arrow)) on the telomere of a single acrocentric chromosome in the transgenic coho strain 5750.
(**D**) Identification of a YY individual in coho salmon using the GH-Y probe (labeled in red). Arrows show the location of the two Y chromosomes in this fish. (For color detail, please see the color plate section.)

Phillips et al. (2004) found two different sequence variants of the internal transcribed spacer of the 5S gene, one of which is associated only with the 5S rDNA on the X chromosome. There are more copies of the 5S gene on the X chromosome, which are thought to be preferentially expressed in oocytes.

FISH with probes to rDNA have also been used to characterize the karyotypes of scallops important to aquaculture. There appear to be at least two types of karyotypes in scallops: one with a haploid number of 16 and the other with a haploid number of 19. An example of a species with $N = 16$ is the Zhikong scallop (*Chlamys farreri*), while the bay scallop (*Argopecten irradians irradians*) has $N = 19$ (Wang et al. 2004). Localization of the major and minor rDNA cistrons in these species showed that *C. farreri* had one locus for the major rDNA and one for the minor rDNA, while *A. irradians irradians* had two major rDNA loci. Two commercial scallop species from Europe (the Great scallop, *Pecten maximus,* and the black scallop, *Mimachlamys varia*) have a karyotype with $2N = 19$ and a single location for both the major and minor rDNA loci (Insua et al. 2006).

Tandem repetitive sequences evolve rapidly, so that centromeric sequences are shared only by closely related species (reviewed in Phillips 2001). For example, in salmonid fishes, there are three different families of centromeric sequences found in all species of the genus *Salvelinus* (Phillips et al. 2002), and one of these is also found in *Oncorhynchus* (Reed et al. 1998; Phillips et al., unpublished). Probes obtained by screening the trout bacterial artificial chromosome (BAC) library with these sequences have been used for chromosome identification in rainbow trout (Phillips et al. 2006; Fig. 5.1B). Another family of centromeric sequences that is useful for chromosome identification has been identified in Atlantic salmon (*Salmo salar*) and clones obtained by screening a BAC library differentiate some of the chromosome pairs (Vinas et al. 2004; Davidsonet al., unpublished). Centromeric sequences have also been identified in Nile tilapia (*Oreochromis niloticus*; Oliveira and Wright 1998) and channel catfish (*Ictalurus punctatus*; Quiniou et al. 2005). It is possible that chromosome-specific probes could be isolated for these species by screening BAC libraries with these satellite DNA sequences.

American (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters have very similar karyotypes with $2N = 20$, but centromeric sequences that are specific to the Pacific oysters allow identification of several specific chromosomes in that species (Wang et al. 2001). FISH with three simple sequence repeats $(GGAT)_4$, $(GT)_7$, and $(TA)_{10}$ gave patterns that were specific for each chromosome in *C. gigas* (Bouilly et al. 2008).

There is a conserved repetitive sequence $(TTTAGGG)_{10}$ found at the telomeres of most vertebrate chromosomes, and this has been found to be present in many fishes. In *O. niloticus*, this sequence has been found at the telomeres of all of the chromosome pairs as well as at two interstitial sites in the largest chromosome pair. This supports the hypothesis that this chromosome pair is the result of two tandem fusions that reduced the chromosome number from $2N = 48$ to $2N = 42$ (Chew et al. 2002).

A dispersed *SalI* repeat in abalone was examined for usefulness in identification of hybrids between *Haliotis fulgens* and *H. rufescens* (Hernandez-Ibarra et al. 2008). The repeat is 87% identical in sequence and distributed over multiple chromosomes in both species. Because of intraspecific variation, it is not useful for hybrid identification.

Transposable elements (TEs) have been localized in several model fish genomes including *Tetraodon nigroviridis* (Crollius et al. 2000; Bouneau et al. 2003; Fischer et al. 2005). There are a larger number of families of TEs in teleosts compared to mammals (Volff 2005). In general, TEs accumulate in heterochromatin regions in fish (reviewed in Dettai et al. 2007). The TE composition of some fish genomes important to aquaculture, including Atlantic salmon (de Boer et al. 2007) and catfish (Nandi et al. 2007), has been reviewed recently.

One application of FISH is to determine whether transgenes have incorporated at single or multiple chromosomal sites and identify the specific sites where they are located. This has been done for growth hormone transgenes in several cell lines of coho salmon (*Oncorhynchus kisutch*). These transgenes appear to have preferentially localized at

either centromeric or telomeric locations (Phillips and Devlin 2010; Fig. 5.1C).

IDENTIFICATION AND CHARACTERIZATION OF SEX CHROMOSOMES

The genetic basis of sex determination has been studied extensively in fishes involved in aquaculture because unisex populations are often preferred by the industry. Male fingerlings are preferred by tilapia growers (Beardmore et al. 2001) and females by salmonid fish farms (Thorgaard et al. 2002). Sex chromosomes have been identified in both salmonid fishes and in tilapia. All salmonid fishes have the XX/XY mode of sex determination (Donaldson and Hunter 1982), while different tilapiine species may have the XX/XY or ZZ/ZW mode (reviewed in Cnaani et al. 2008).

The Nile tilapia, *O. niloticus,* has the sex-determining (SD) locus on linkage group 1 (LG1) and the male is heterogametic (XX-XY system), while in *O. aureus* from Israel, *O. karongae* and *Tilapia mariae*, the SD locus is on LG3 and the female is heterogametic (WZ/ZZ system; Cnaani et al. 2008). Other species such as *O. mossambicus* and the Egyptian strains of *O. niloticus* (Lee et al. 2003) and *O. aureus* have a more complex situation with markers on both linkage groups linked with phenotypic sex. This is probably due to a sex-linked lethal in *O. mossambicus.* Although the sex chromosomes of *O. niloticus* are not morphologically distinguishable, FISH with repetitive DNAs shows that the short arm of the X and Y chromosome differ in the amount of repetitive DNA present (Ferreira and Martins 2008).

In salmonid fishes, autosomal genetic linkage groups corresponding to whole chromosome arms are conserved in all species studied to date, but the SD gene is on a different linkage group in each species (Woram et al. 2003). The male-specific region on the Y chromosome appears to be small in each species, but male-specific markers are conserved in members of the genus *Oncorhynchus,* suggesting that this small region has transposed to different chromosomes in different species (Devlin et al. 2001; Brunelli et al. 2008). This hypothesis is supported by the fact that there is a male-specific growth hormone pseudogene (GH-Y) related to growth hormone 2 (Du et al. 1993; Zhang et al. 1999) common to all Pacific salmon.

In both salmonids and tilapia, BACs containing sex-linked markers have been isolated and used as probes in FISH experiments. These experiments have shown that BACs containing specific markers are found in the same order as the genetic linkage map, but with more space between them on the cytogenetic map than on the genetic linkage map. Experiments in tilapia showed that LG3 is the largest chromosome in the karyotype and that LG1 is a smaller chromosome. FISH experiments with different sex-linked BACs showed that there is a large region of approximately 50 megabases (Mb) taking up most of the long arm of the chromosome of the largest chromosome pair in which recombination is suppressed. In rainbow trout, the sex chromosome pair is the only subtelocentric chromosome pair in the karyotype (Thorgaard 1977), and recombination is suppressed in a region of at least 20 Mb over much of the long arm of the chromosome (Phillips et al. 2009a).

The sex chromosome pairs in different species of salmonid fishes have been identified or confirmed using FISH with sex-linked markers. In the case of the *Oncorhynchus* species, probes to male-specific markers such as GH-Y have been used to determine where on the chromosome the SD is located. All of the salmonid species, with the exception of two sister species pairs cutthroat trout (*Oncorhynchus clarki*)/rainbow trout (Alfaqih et al. 2008) and brook trout (*Salvelinus fontinalis*)/lake trout(*Salvelinus namaycush*) (Phillips et al. 2002), have different sex chromosomes. In lake trout and brook trout, the sex chromosome pair is the only large submetacentric chromosome, and the SD is found on the short arm that has a block of heterochromatin at the end (Phillips and Ihssen 1985; Phillips et al. 2002). In Arctic charr (*Salvelinus alpinus*), the SD is on a different linkage group from lake trout (Woram et al. 2003). In Atlantic salmon, the sex chromosome pair is the second largest chromosome in the karyotype of the European strain and the chromosome arm containing the SD corresponds to the short arm of the chromosome 17 (Omy 17, LG 29) in rainbow trout. The SD locus appears to be located adjacent to a large block of heterochromatin at the end of the long arm of the Atlantic salmon

chromosome (Artieri et al. 2006). In chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*), the sex chromosomes are large acrocentric chromosomes (Stein et al. 2001; Phillips et al. 2005), corresponding to Omy 15 (LG7q) and Omy 8 (LG23q) in rainbow trout, respectively (Phillips et al. 2005; McClelland and Naish 2008). In both of these species, the SD appears to be located on the short arm, and in Chinook salmon, there is a large block of repetitive DNA in the middle of the long arm of the chromosome (Stein et al. 2001). In pink salmon and chum salmon, the sex chromosomes are smaller acrocentrics and again the male-specific region (as identified using a probe to GH-Y) is found on the small short arms of these chromosomes (Phillips et al. 2007). In sockeye salmon, a Y-autosomal fusion has occurred so that males have $2N = 57$ and females $2N = 58$ (Thorgaard 1978), and GH-Y is not present.

The GH-Y probe has been used to identify YY coho salmon (Fig. 5.1D), which make up 25% of the progeny from matings between sex-reversed XY fish and normal XY males (LK Park, personal communication). These fish can be mated with normal females to produce all-male progeny.

Recently, we used FISH to examine the sex chromosomes in the apparent XY-female fall chinook in the Sacramento, California, drainage. The usually male-specific chinook salmon markers Oty1 (Devlin et al. 1998) and GH-Y (Du et al. 1993) were found in 30% of female chinook over a large region in northern California (Williamson et al. 2008). We examined the sex chromosomes in these females and crosses, including them as parents, and confirmed that they had one sex chromosome with Oty1 and GH-Y sequences, but these were present on these modified Y* chromosomes in smaller amounts than in normal males. When crossed with normal males, these XY* females produced OtY1 and GH-Y positive offspring in a 3:1 ratio, but males and females in a 1:1 ratio. Half of the females inherited the Y* chromosome with reduced OtY1 and GH-Y from the female parent. The most parsimonious explanation is that the Y chromosomes with smaller amounts of OtY1 and GH-Y have a deletion of the SD locus. It is also possible that crossing over between the X and Y resulted in an X chromosome with the male-specific markers. In any case, these fish have been stocked over a wide area of inland California, so the OtY1 and GH-Y markers cannot be used to identify males in this region.

CHARACTERIZATION OF INTERSPECIFIC HYBRIDS AND CHROMOSOME SET MANIPULATED FINFISH

Whole chromosome painting can be used to analyze interspecific hybrids. Briefly, genomic DNA is isolated from each species and labeled using a fluorescent dye of a different color (Snowdon et al. 1997). Application of this technique to hybrids produced in the laboratory between masu salmon (*Oncorhynchus masou*) females and rainbow trout males showed that the trout chromosomes were eliminated, but only whole masu salmon chromosomes were maintained in embryonic cells. However, fragments of the trout chromosomes were found in micronuclei in these embryos (Fujiwara et al. 1997). Analysis of natural hybrids between Atlantic salmon and brown trout using a probe to histone DNA showed that the hybrids contained the specific chromosomes containing the histone genes from both species. Other applications of FISH to manipulated finfish are reviewed in Ocaleowicz et al. (2007).

ASSIGNMENT OF GENETIC LINKAGE GROUPS TO SPECIFIC CHROMOSOMES (GENOME MAPPING)

In order to assign genetic linkage groups to chromosomes using FISH, it is necessary to have large clones such as cosmid or BAC clones containing specific genetic markers that have been placed on a genetic linkage map. Currently, BAC libraries are available for catfish (Quiniou et al. 2003), three salmonid fishes, including Atlantic salmon (Thorsen et al. 2005), rainbow trout (Katagiri et al. 2001; Phillips et al. 2003; Palti et al. 2004), and chinook salmon (Devlin, personal communication), tilapia (Katigiri et al. 2005), barramundi (Wang et al. 2008), and oysters (Gaffney, personal communication). Cosmid libraries are available for additional species, including coho salmon (Devlin, personal communication) and Arctic charr (Davidson, personal communication). Usually, BAC clones will hybridize to closely related species, so that additional libraries do not have to be prepared for each species. For example, rainbow trout BAC clones can

be used on coho and chinook salmon chromosomes (Phillips, unpublished).

Linkage groups have been assigned to all of the chromosome pairs and chromosome arms in rainbow trout (Phillips et al. 2006) and Atlantic salmon (Phillips et al. 2009b) using FISH with BAC clones containing markers on the genetic linkage maps of these species (Fig. 1B). Comparisons of the genetic linkage maps and cytogenetic maps have shown that there is conservation of large syntenic blocks comprising entire chromosome arms between the two species.

The ancestor of salmonid fishes underwent a whole-genome duplication (WGD) prior to adaptive radiation (Allendorf and Thorgaard 1984), and most of the species have approximately 100 chromosome arms (Phillips and Rab 2001). For example, if one ignores chromosome arms containing entirely rDNA and assumes that the smallest metacentric (bi-armed) chromosome pair in the rainbow trout karyotype (which is smaller than the largest acrocentric (uni-armed) chromosome pair) underwent an inversion and should be counted as a uni-armed chromosome pair, then the karyotypes of all members of the genus *Oncorhynchus* have exactly 100 chromosome arms (Phillips et al. 2009b). There are also approximately 100 chromosome arms in the karyotypes of most members of the genus *Salvelinus* and in brown trout (*Salmo trutta*). Arctic charr and brown trout have one chromosome pair that appears to be a tandem fusion, reducing the number of chromosome arms to 98 in these species. The Atlantic salmon (*S. salar*) karyotype is a major exception to this rule with 74 chromosome arms in the European strain and 72 in the North American strain. However, the presence of 12 large acrocentric chromosomes with repetitive DNA blocks in the center of the arms suggested that many tandem fusions have occurred in the Atlantic salmon karyotype (Phillips and Rab 2001). This has been confirmed by the integration of the genetic and cytogenetic maps of Atlantic salmon and rainbow trout (Phillips et al. 2009b). Each of these large acrocentric chromosomes corresponds to two rainbow trout chromosome arms separated by a block of repetitive DNA in the center of the chromosome arm (Phillips et al. 2009b). In addition, the largest chromosome pair in the European strain of Atlantic salmon has undergone a tandem fusion in the long arm, which is marked by a band of repetitive DNA. There are three chromosome arms from rainbow trout that correspond with this chromosome in Atlantic salmon.

The Atlantic salmon genome is scheduled to be sequenced starting in 2009 (Davidson, personal communication) and the rainbow trout genome will probably be sequenced within a couple of years (Palti, personal communication). The integration of the cytogenetic and genetic maps of these two species will be very helpful in annotating their genomes. Correlation of the genetic and cytogenetic maps of the other salmonids with the rainbow trout maps will also be very helpful in transferring information from these genome projects to the other species, a number of which are being important in aquaculture.

We are currently assigning the linkage groups of coho salmon and chinook salmon to specific chromosomes using FISH with BAC clones derived from rainbow trout (Phillips et al., unpublished). The karyotypes of these two species have 100 chromosome arms, and we should be able to correlate them completely with rainbow trout. This will be very useful since there are several genetic maps of the trout genome that are considerably more detailed than the ones for these salmon genomes and a genome sequence may be available for rainbow trout in the next few years. So far, the syntenic blocks usually contain markers in the same order, suggesting that very few intrachromosomal rearrangements have occurred.

IDENTIFICATION OF PATHOGENS IN CULTURED SHELLFISH, FISH, AND WASTEWATER GENERATED BY AQUACULTURE

One of the problems associated with the expansion of the marine aquaculture is the accumulation of nitrogenous compounds, especially ammonia, which are toxic to aquatic animals above certain concentrations (Alcarez et al. 1999). Chemolithotrophic bacteria are responsible for ammonia oxidation and its removal from the ecosystem. Studies of microbial communities in wastewater from aquaculture using molecular approaches have enabled a better understanding of the organisms that are found in these communities (reviewed in Amman et al. 2001). The technique of FISH enables investigators to analyze these complex communities in situ in natural and

engineered facilities. For example, studies of wastewater from a shrimp farm using PCR with universal primers to bacterial 16S rDNA found both ammonia oxidizing bacteria and nitrifying bacteria, including several novel taxa from these groups (Paungfoo et al. 2007). FISH was used to partially quantitate the number of specific bacterial species found in different parts of the system. In another application, microbial communities in the biological filters and waste sludge components of a recirculating water aquaculture system were examined to determine the presence of anaerobic ammonium-oxidizing bacteria. PCR with the 16S rDNA primers on sludge material revealed a diverse microbial community, including one type with sequence similarity to known anaerobic ammonium-oxidizing bacteria. Then, a fluorescent probe specific to this bacteria was produced and the presence and distribution of it on the biofilms was confirmed using FISH (Tal et al. 2006).

Another application of FISH to aquaculture has been in identification of pathogens present in cultured shellfish. This can be important in species under long-term cultivation, such as marine sponges. For example, explants were derived from the cultivated marine sponge *Geodia barretti,* slides were made, and FISH was used to confirm presence of Proteobacteria in the explants (Hoffman et al. 2006). To summarize, FISH is being used both in the localization and identification of pathogens in aquaculture animals and in situ in aquaculture facilities that used to grow them.

FUTURE APPLICATIONS

Future applications of FISH include comparative genome mapping in related species of interest to aquaculture, localization of genes on meiotic chromosomes, and development of fiber FISH for high-resolution mapping of large insert clones directly onto the DNA fibers of specific genomic regions (H-UG; Weier 2001). Genome sequences of many aquatic organisms will be generated in the next few years, and the whole genome assemblies will inevitably have a number of gaps. Fiber FISH can be used to map the location and size of these gaps. It will also be useful for genetic mapping of regions of low recombination, which often also contain large tracks of repetitive DNAs (e.g., regions near centromeres and large portions of the sex chromosomes), where sequencing is difficult.

Another future application is the combination of FISH with immunoassays to detect specific DNA sequences that are bound to specific proteins. Immuno-FISH has been used in plants to reveal the association of histone modifications with specific genomic regions (reviewed in Jiang and Gill 2006), and this technique should be useful in gene expression studies.

REFERENCES

Alcarez G, Espinoza V, Vanegas C, and Carrara XC. 1999. Acute effect of ammonia and nitrate on respiration of *Penaeus setiferus* post larvae under different oxygen levels. J World Aquacult Soc. 30: 98–106.

Alfaqih M, Phillips RB, Wheeler PA, and Thorgaard GH. 2008. The cutthroat trout Y chromosome is conserved with that of rainbow trout. Cytogenet Genome Res. 121: 255–259.

Allendorf FW and Thorgaard GH. 1984. Tetraploidy and the evolution of salmonid fishes. In: Evolutionary Genetics of Fishes (ed. Turner BJ). Plenum Press, New York. pp 1–53.

Amman R, Fuchs BM, and Behrens S. 2001. Identification of microorganisms by fluorescence *in situ* hybridization. Curr Opin Biotech. 12: 231–236.

Artieri CG, Ng S, Mitchell L, Danzmann RG, Phillips RB, Schein J, Koop BF, and Davidson WS. 2006. Identification of the sex-determining locus of Atlantic salmon (*Salmo salar*) on chromosome 2. Cytogenet Genome Res. 112(1–2): 152–159.

Beardmore JA, Mair GC, and Lewis RL. 2001. Monosex male production in finfish as exemplified by tilapia: applications, problems and prospects. Aquaculture. 197: 283–301.

Bouilly K, Chaves R, Leitao A, Benabdelmouna A, and Guedes-Pinto H. 2008. Chromosomal location of simple sequence repeats in the Pacific oyster (*Crassostrea gigas*): $(GGAT)_4$, $(GT)_7$ and $(T)_7$ chromosome patterns. J Genet. 87: 119–125.

Bouneau L, Fischer C, Ozouf-Costaz C, Froschauer A, Jaillon O, Coutanceau JP, Körting C, Weissenbach J, Bernot A, and Volff JN. 2003. An active non-LTR retrotransposon with tandem structure in the compact genome of the pufferfish *Tetraodon nigroviridis*. Genome Res. 13: 1686–1695.

Brunelli JP, Wertzler KJ, Sundin K, and Thorgaard GH. 2008. Y-specific sequences and polymorphisms in rainbow trout and chinook salmon. Genome. 51: 739–748.

Chew JS, Oliveria C, Wright JM, and Dobson MJ. 2002. Molecular and cytogenetic analysis of the telomeric (TTAGGG)n repetitive sequences in the Nile tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae). Chromosoma. 111(1): 45–52.

Crollius H, Jaillon O, Dasilva C, Ozourf-Costz C, Fizames C, Fisher C, Bouneau L, Billault A, Questier F, Saurin W, Bernot A, and Weissenbach J. 2000. Characterization and repeat analysis of the compact genome of the freshwater pufferfish *Tetraodon nigroviridis*. Genome Res. 10: 939–949.

Cnaani A, Lee B-Y, Zilberman N, Ozouf-Costz C, Hulata G, Ron M, D'Hont A, Baroiller J-F, Cotta H, Penman DJ, Tornasino T, Coutanecau J-P, Pepey E, Shirak A, and Kocher TD. 2008. Genetics of sex determination in tilapiine species. Sex Dev. 2: 43–54.

de Boer JG, Yazawa R, Davidson WS, and Koop BF. 2007. Bursts and horizontal evolution of DNA transposons in the speciation of pseudotetraploid salmonids. BMC Genomics 8: 422.

Dettai A, Bouneau L, Fischer C, Schultheis C, Schmidt C, Zhou Q, Coutaneau J-P. Ozouf-Costz C, and Volff J-N. 2007. FISH analysis of fish transposable elements: tracking down mobile DNA in teleost genomes. In: Fish Cytogenetics (eds. Pisano E, Ozouf-Costaz C, Foresti F, and Kapoor BG). Chapter 4.4. Science Publishers, Enfield, NH. pp 361–383.

Devlin RH, Stone GW, and Smailus DE. 1998. Extensive direct tandem organization of a long repeat DNA sequence on the Y chromosome of chinook salmon (*Oncorhynchus tshawytscha*). J Mol Evolution. 46: 277–287.

Devlin RH, Biagi CA, and Smailus DE. 2001. Genetic mapping of Y-chromosomal DNA markers in Pacific salmon. Genetica. 111: 43–58.

Donaldson EM and Hunter GA. 1982. Sex control in fish with particular reference to salmonids. Can J Fish Aquat Sci. 39: 99–110.

Du SJ, Devlin RH, and Hew CL. 1993. Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-U. DNA Cell Biol. 12: 739–751.

Ferreira IA and Martins C. 2008. Physical chromosome mapping of repetitive DNA sequences in Nile tilapia *Oreochromis niloticus:* evidences for a differential distribution of repetitive elements in the sex chromosomes. Micron. 39: 411–418.

Fischer C, Bouneau L, Coutanceau JP, Weissenbach J, Ozouf-Costaz C, and Volff JN. 2005. Diversity and clustered distribution of retrotransposable elements in the compact genome of the pufferfish *Tetraodon nigroviridis*. Cytogenet Genome Res. 110: 522–536.

Foresti F. 2000. Biotechnology and fish culture. Hydrobiologia. 420: 45–47.

Fujiwara A, Abe S, Yamaha E, Yamazaki F, and Yoshida MC. 1997. Uniparental chromosome elimination in the early embryongenesis of the inviable salmonid hybrids between masu salmon female and rainbow trout male. Chromosome. 106: 44–52.

Hernandez-Ibarra NK, Leitch AR, Cruz P, and Ibarra AM. 2008. Fluorescent *in situ* hybridization and characterization of the *Sal*I family of satellite repeats in the *Haliotis* L. species (abalone) of the Northeast Pacific. Genome. 51: 570–579.

Hoffman F, Rapp HT, and Reitner J. 2006. Monitoring microbial community composition by fluorescence *in situ* hybridization during the cultivation of the marine cold-water sponge *Goedia barrettia*. Marine Biotech. 8: 373–379.

Insua A, Lopez-Pinon MJ, Freire R, and Mendez J. 2006. Karyotype and chromosomal location of 18S-28S and 5S ribosomal DNA in the scallops *Pecten maximus* and *Mimachlamys varia* (*Bivalvia Pectinidae*). Genetica. 126: 291–301.

Jiang J and Gill BS. 2006. Current status and future applications of fluorescence *in situ* hybridization (FISH) in plant genome research. Genome. 49: 1057–1058.

Katagiri T, Asakawa S, Minagawa S, Shimizu N, Hirono I, and Aoki T. 2001. Construction and characterization of BAC libraries for three fish species: rainbow trout, carp and tilapia. Anim Genet. 32: 200–204.

Katigiri T, Kidd C, Tomasino E, Davis JT, Wishon C, Stern JE, Carleton KL, Howe AE, and Kocher TD. 2005. A BAC-based physical map for the Nile tilapia genome. BMC Genomics. 6: 89.

Lee BY, Hulata G, and Kocher TD. 2003. Identification of a sex-determining region in Nile tilapia (*Oreochromis niloticus*). Anim Genet. 38: 85–86.

Martins C. 2007. Chromosomes and repetitive DNAs: a contribution to the knowledge of the fish genome. In: Fish Cytogenetics (eds. Pisano E, Ozouf-Costz C, Foresti F, and Kapoor BG). Chapter 4.4. Science Publishers, Enfield, NH. pp 421–453.

Martins C and Galetti PM. 2001. Two 5S rDNA arrays in neotropical fishes: is it a general rule for fishes? Genetica. 111: 439–446.

McClelland EK and Naish KA. 2008. A genetic linkage map for coho salmon (*Oncorhynchus kisutch*). Anim Genet. 39: 169–179.

Nandi S, Peatman E, Xu P, Wang S, Li P, and Liu Z. 2007. Repeat structure of the catfish genome: a genomic and transciptomic assessment of Tc1-like transposon elements in channel catfish (*Ictalurus punctatus*). Genetica. 131: 81–90.

Ocaleowicz K, Jankun M, and Luczynski M. 2007. Cytogenetic characteristics of interspecific hybrids and chromosome set manipulated finfish. In: Fish Cytogenetics (eds. Pisano E, Ozouf-Costaz C, Foresti F, and Kapoor BG). Chapter 3.2. Science Publishers, Enfield, NH. pp 280–332.

Oliveira C and Wright JM. 1998. Molecular cytogenetic analysis of heterochromatin in the chromosomes of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae). Chromosome Res. 6: 205–211.

Palti Y, Gahr SA, Hansen JD, and Rexroad CE 3rd. 2004. Characterization of a new BAC library for rainbow trout: evidence for multi-locus duplication. Anim Genet. 35: 130–136.

Paungfoo C, Prasertsan P, Burrell PC, Intrasungkha N, and Blackall LL. 2007. Nitrifying bacterial communities in an aquaculture wastewater treatment system using fluorescence *in situ* hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis. Biotechnol Bioeng. 97: 985–990.

Phillips RB. 2001. Application of fluorescence *in situ* hybridization (FISH) to fish genetics and genome mapping. Mar Biotechnol. 3: 5145–5152.

Phillips RB and Ihssen PE. 1985. Identification of sex chromosomes in lake trout (*Salvelinus namaycush*). Cytogenet Cell Genet. 39(1): 14–18.

Phillips RB, Morasch MR, Park LK, Naish KA, and Devlin RH. 2005. Identification of the sex chromosome pair in coho salmon (*Oncorhynchus kisutch*): lack of conservation of the sex linkage group with chinook salmon (*Oncorhynchus tschawytscha*). Cytogenet Genome Res. 111: 166–170.

Phillips RB and Reed KM. 1996. Application of fluorescence in situ hybridization (FISH) to fish genetics. Aquaculture. 140: 197–216.

Phillips RB and Rab P. 2001. Chromosome evolution in the Salmonidae (Pisces): an update. Biol Rev. 76: 1–25.

Phillips RB, Matsuoka MP, and Reed KM. 2002. Characterization of charr chromosomes using fluorescence *in situ* hybridization. Environ Biol Fishes. 64: 223–228.

Phillips RB, Zimmerman A, Noakes M, Palti Y, Morasch M, Eiben L, Ristow S, Thorgaard GH, and Hansen JD. 2003. Physical and genetic mapping of the rainbow trout major histocompatibility regions: evidence for duplication of the class I region. Immunogenetics. 55: 561–569.

Phillips RB, Nichols KM, DeKoning JJ, Morasch MR, Keatley KA, Rexoad C, Gahr S, Danzmann RG, Drew RE, and Thorgaard GH. 2006. Assignment of rainbow trout linkage groups to specific chromosomes. Genetics. 174: 1661–1670.

Phillips RB, DeKoning JJ, Morasch MR, Park LK, and Devlin RH. 2007. Identification of the sex chromosome pair in chum salmon (*Oncorhynchus keta)* and pink salmon (*Oncorhynchus gorbuscha*). Cytogenet Genome Res. 116: 298–304.

Phillips RB. 2007. Application of fluorescence in situ hybridization (FISH) to genome mapping in fishes. In: Fish Cytogenetics (eds. Pisaro E, Ozouf-Costaz C, Foresti F, and Kapoor BG). Chapter 4.5. Academic Press, New York. pp 455–473.

Phillips RB, DeKoning JJ, Ventura AB, Nichols KM, Drew RE, Chaves LD, Reed KM, Felip A, and Thorgaard GH. 2009a. Recombination is suppressed over a large region of the rainbow trout Y Chromosome. Anim Genet. 40: 925–932.

Phillips RB, Keatley KA, Morasch MR, Ventura AB, Lubieniecki, KP, Koop, BF, Danzmann RG, and Davidson WS. 2009b. Assignment of Atlantic salmon (*Salmo salar*) linkage groups to specific chromosomes: conservation of large syntenic blocks corresponding to whole chromosome arms in rainbow trout (*Oncorhynchus mykiss*). BMC Genet. 10: 46.

Phillips RB and Devlin RH. 2010. Integration of growth hormone gene constructs in transgenic strains of coho salmon (*Oncorhynchus kisutch*) at centromeric or telomeric sites. Genome. 53: 79–82.

Phillips RB, Noakes MA, Morasch M, Felip A, and Thorgaard GH. 2004. Does differential selection on the 5S rDNA explain why the rainbow trout sex chromosome heteromorphism is not linked to the SEX locus? Cytogenet Genome Res. 105: 122–125.

Porto-Foresti F, Oliveira C, Tabata YA, Rigolino MG, and Foresti F. 2007. NOR markers in the identification and management of cultured fish species: the case of rainbow trout stocks reared in Brazil. In: Fish Cytogenetics (eds. Pisano E, Ozouf-Costaz C, Foresti F, and Kapoor BG). Science Publishers, Enfield, NH. Chapter 3.3. pp 333–357.

Quiniou SM, Katagiri T, Miller NW, Wilson M, Wolters WR, and Waldbieser GC. 2003. Construction and characterization of a BAC library from a gynogenetic channel catfish *Ictalurus punctatus*. Genet Select Evol. 35: 673–683.

Quiniou SM, Wolters WR, and Waldbieser GC. 2005. Localization of Xba repetitive elements to channel catfish (*Ictalusrus punctatus*) centromeres via fluorescence *in situ* hybridization. Anim Genet. 36: 353–354.

Reed K M, Dorschner MO, and Phillips RB. 1998. Characteristics of two salmonid repetitive DNA families in rainbow trout (*Oncorhynchus mykiss*). Cytogenet Cell Genet. 79: 184–187.

Snowdon RJ, Kohler W, Friedt W, and Kohler A. 1997. Genomic *in situ* hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet. 95: 1320–1324.

Stein JD, Phillips RB, and Devlin RH. 2001. Identification of sex chromosomes in chinook salmon (*Oncorhynchus tshawytscha*). Cytogenet Cell Genet. 92: 108–110.

Tal Y, Watts JEM, and Schreier HJ. 2006. Aerobic ammonium-oxidizing (Anammox) bacteria and associated activity in fixed-film biofilters of a marine re-circulating aquaculture system. Appl Environ Microb. 72: 2896–2904.

Thorsen J, Zhu B, Frengen E, Osoegawa K, de Jong PJ, Koop BF, Davidson WS, and Høyheim B. 2005. A highly redundant BAC library of Atlantic salmon (*Salmo salar*): an important tool for salmon projects. BMC Genomics. 6: 50.

Thorgaard GH. 1977. Heteromorphic sex chromosomes in male rainbow trout. Science. 196: 900–902.

Thorgaard GH. 1978. Sex chromosomes in the sockeye salmon: a Y-autosomal fusion. Can J Genet Cytol. 20: 349–354.

Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattari SL, Ristow SS, Hansen JD, Winton JR, Bartholomew JL, Nagler JJ, Walsh PJ, Vijayan MM, Devlin RH, Hardy RW, Overturf KE, Young WP, Robison BD, Rexroad C, and Palti Y. 2002. Status and opportunities for genomics research with rainbow trout. Comp Biochem Physiol B Biochem Mol Biol. 133(4): 609–646.

Vinas A, Abuin M, Pardo BG, Martinez P, and Sanchez L. 2004. Characterization of a new HpaI centromeric satellite DNA in *Salmo salar*. Genetica. 121: 81–87.

Volff JN. 2005. Genome evolution and biodiversity in teleost fish. Heredity. 94: 280–294.

Wang CM, Lo LC, Feng F, Gong P, Li J, Zhu ZY, Lin G, and Yue GH. 2008. Construction of a BAC library and mapping BAC clones to the linkage map of Barramundi, *Lates calcarifer*. BMC Genomics 25: 139.

Wang Y, Xu Z, and Guo X. 2001. A centromeric satellite sequence in the Pacific oyster (*Crassostrea gigas* Thunberg) identified by fluorescence *in situ* hybridization. Mar Biotechnol. 3: 486–492.

Wang Y, Xu Z, and Guo X. 2004. Differences in the 5S rDNA chromosome divide the Asian Pacific and Atlantic Pacific species of *Crassostrea* (Bivalvia Mollusca). Biol Bull. 206: 46–54.

Weier H-UG. 2001. DNA fiber mapping techniques for assembly of high resolution physical maps. J Histoch Cytochem. 49: 939–948.

Williamson KS, Phillips RB, and May B. 2008. Characterization of a chromosomal rearrangement responsible for producing apparent XY female fall chinook salmon in California. J Hered. 99: 483–490.

Woram RA, Gharbi K, Sakamoto T, Hoyheim B, Holm L-E, Naish K, McGowan C, Ferguson MM, Phillips RB, Stein J, Guyomard R, Ciarney M, Taggart J, Powell R, Davidson W, and Danzmann RG. 2003. Comparative genome analysis of the primary sex determining locus in salmonid fishes. Genome Res. 13: 272–280.

Zhang Q, Nakayama I, Fujiwara A, Kobayashi T, Oohara T, Masaoka T, Kitamura S, and Devlin RH. 1999. Identification of a sex-linked GH pseudogene in one of two species of Japanese salmon (*Oncorhynchus masou* and *Oncorhynchus rhodurus*). Aquaculture. 173: 65–72.

Part 3
Fish Health

Chapter 6

The Application of Genomics, Proteomics, and Metabolomics to Studies of Fish Health

Stewart C. Johnson and Laura L. Brown

INTRODUCTION

Genomics, proteomics, and metabolomics, used alone or in combination with each other and/or with more traditional methods, are technologies that are rapidly transforming many areas of biological and biomedical research. Although these technologies have been widely used in the study of diseases of higher vertebrates, as well as in the development of new therapeutants and vaccines, they are only now being routinely used to support research in fish health.

Increased use of these technologies in fish health research is due to many factors, including the fish health research community recognizing the tremendous advances that have been made in other areas of health research through their use, development of commercial kits/reagents that have made these technologies simpler to apply, and lowered costs of some of these activities, for example, the development of high-throughput and less expensive sequencing technology.

These technologies allow for the simultaneous study of many genes, proteins, or metabolites, as well as the interactions that occur between them. Our ability to generate these large and very complex data sets brings with it a new set of problems to be faced by the fish health research community in the areas of data management, analysis (bioinformatics and statistics), storage, and archiving.

Genomics

For the purpose of this chapter, genomics has been defined in its broadest sense as the description, definition, and characterization of the genetic makeup of an organism. This includes the area of functional genomics, which focuses on the study of the dynamic aspects of genes (e.g., transcription and translation) and the biological function of genes and their products. As will be demonstrated, we are presently experiencing a rapid increase in the availability of genomic information for pathogens of aquatic animals. Improvements in sequencing technologies, which allow for more rapid sequencing with significantly lower costs, have made this possible. With respect to hosts, we still have, for most species, relatively limited genomic resources. To date, genomic studies on hosts have been limited primarily to expressed sequence tags (ESTs) projects that have been

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

conducted on a variety of scales ranging from a few larger projects such as the consortium for Genomics Research on All Salmonids Project (cGRASP) to a large number of smaller programs on other fish species. From these projects, there have been developed a number of genomic tools such as microarrays (reviewed in Dios et al. 2008; Goetz and MacKenzie 2008; Martin et al. 2008).

Proteomics

The proteome can be defined as the entire complement of proteins expressed by a genome, cell, tissue, or organism at a given time under defined conditions. The field of proteomics allows for the study of proteins that make up the proteome and provides information on their expression, posttranslational modifications, interactions, organization, and functions. As we will see, understanding transcriptional response of pathogens and hosts has proven to be extremely valuable in our understanding of fish diseases. However, we will always have an incomplete understanding of these processes until we understand how the observed transcriptional responses are reflected in proteomes, as it is proteins and their interactions that are responsible for regulation of almost all aspects of cellular function. Proteomics provides information on whether gene transcription is reflected as protein expression, whether the resulting protein(s) undergoes posttranslational modifications that are often necessary for their activity, and whether gene transcripts are responsible for the production of more than one protein through processes such as alternative splicing.

It is beyond the scope of this chapter to provide a detailed review of proteomic methods, but interested readers should consult the following recent reviews that describe proteomic applications for the study of bacterial membranes and fish (Poetsch and Wolters 2008; Martyniuk and Denslow 2009, respectively). With respect to applications in fish health research, the proteomics method that has seen the widest use is two-dimensional polyacrylamide gel electrophoresis (2D PAGE) with or without further protein characterization by mass spectrophotometry. In 2D PAGE, proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS-PAGE in the second dimension. Separation of proteins in this manner allows for the resolution of complex mixtures of proteins, including features of proteins such as posttranslational modifications. As mentioned, proteins separated by 2D PAGE can be isolated and further characterized by mass spectrophotometric analysis, as well as by other techniques as described below.

Although there is great value in proteomics approaches, there are some significant problems that can limit their application especially in poorly studied organisms. Characterization and identification of peptide sequences determined during proteomic analysis relies on the availability of protein databases for comparison. Sequencing of fish pathogens and related species has greatly improved our ability to apply proteomics techniques in the study of pathogens. The lack of protein databases for fish makes the application of proteomics more difficult. In the case of 2D PAGE, the analysis of proteomes is often less than satisfactory due to poor reproducibility between gels; this is usually attributed to differences in laboratory procedures (human factors) and/or reagents. In addition, the preparatory methods such as the method used to solubilize proteins can lead to different proteome maps for the same samples. Furthermore, proteins that are present in low abundance often cannot be identified.

Metabolomics

For the purpose of this chapter, metabolomics is defined as the systematic study of the complete set of small (<1 kDa) metabolites present in a cell or tissue under a particular set of conditions. The metabolome refers to all the metabolites that can be present within an organism under a particular set of conditions. Metabolite profiles have been determined using nuclear magnetic resonance (NMR) spectroscopy—and less commonly by mass spectrometry-based methods. These methods used alone, and more recently in combination, have been used to identify biomarkers associated with infectious and noninfectious disease and nutritional status especially in humans (reviewed in Orešič 2009). To date, metabolomics has seen its widest application in studies of fish physiology, primarily studies of the response to various toxicants (reviewed in Samuelsson and Larsson 2008). There are relatively

Table 6.1. Viruses Within the Family Iridoviridae for Which There Are Completed Genomic Sequences (data from Entrez Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez).

Virus Name	Genus	Accession Number	References
Singapore grouper iridovirus (SGIV)	*Ranavirus*	AY521625	Song et al. 2004; Tsai et al. 2005
Lymphocystis disease virus (LDV)	*Lymphocystivirus*	NC_001824 NC_005902	Tidona and Darai 1997; Zhang et al. 2004
Infectious spleen and kidney necrosis iridovirus (ISKNV)	*Megalocytivirus*	NC_003494	He et al. 2001
Red seabream iridovirus (RSIV)	*Megalocytivirus*		Kurita et al. 2002
Orange-spotted grouper iridovirus (OSGIV)	*Megalocytivirus*	AY894343	Lu et al. 2005
Large yellow croaker iridovirus (LYCIV)	*Megalocytivirus*	AY779031	Chen and Wang, unpublished sequence in GenBank
Rock bream iridovirus (RBIV)	Unclassified	AY532606	Do et al. 2004

few examples of its application in other areas of fish biology and fish health research, as reviewed below.

This review focuses on the application of these and related technologies to study viruses of the family Iriodoviridae and the bacterium *Aeromonas salmonicida* and their interactions with their fish hosts.

STUDIES OF PATHOGEN BIOLOGY

Viral Pathogens—Iridoviridae

The importance of viral diseases in fish, along with the small sizes of viral genomes and their simple organization, has resulted in a large amount of genomic information for fish viruses. This information includes complete genomic sequences for most economically important viruses, as well as large numbers of partial and complete sequences for a variety of genes. These data are used widely by virologists and other fish health researchers to support studies in numerous areas, including taxonomic and evolutionary studies, investigation of host–pathogen interactions, development of molecular diagnostics, and development of vaccines including both subunit and DNA vaccines (see chapters in this book by Wu et al. (Chapter 9) and Leong et al. (Chapter 10)).

Genomic Studies

To demonstrate the value of genomic resources to the study of fish viruses, we have used as an example the iridoviruses. Iridoviruses infect and cause serious disease in invertebrates and poikilothermic vertebrates, including fish.

To date, full genome sequences are available for seven species of fish iridoviruses belonging to the genera *Ranavirus*, *Lymphocystivirus,* and *Megalocytivirus* (Table 6.1). In addition to these full genome sequences, there are large numbers of sequences for a variety of genome regions and specific genes for iridoviruses isolated from numerous other fish host species. This sequence information, and sequence information from related irodiviruses, has been used to conduct detailed investigations into many aspects of the biology, physiology, and host–pathogen interactions of these viruses, as well as to devise molecular detection methods and novel treatment strategies and vaccines. As examples, this information was used to examine taxonomy and phylogenetic relationships between iridoviruses (He et al. 2001; Song et al. 2004; Zhang et al. 2004; Do et al. 2005; Tsai et al. 2005; Kvitt et al. 2008; Shinmoto et al. 2009) and to conduct molecular epizootiological studies (Go et al. 2006; Wang et al. 2007). Eaton et al. (2007) conducted a comparative genomic analysis

of the genomes of iridoviruses of fish and other animals. From this analysis, they were able to produce updated gene annotations and to redefine the group of core genes shared by all iridoviruses to include 26 genes. These core genes are highly valuable with respect to understanding the phylogenetic relationship between iridoviruses, as well as aspects of their biology, for example, replication. With respect to the fish iridoviruses, those authors predicted based on their analysis that the orange-spotted grouper iridovirus and the rock bream iridovirus (RBIV), which are species within the Megalocytivirus, share an identical complement of genes.

Molecular Diagnostic Test Development

Using sequence data, a number of molecular diagnostics tests including in situ hybridization and PCR techniques have been developed using a variety of genes to screen for virus and/or to examine the appearance of, and tissue distribution of, virus in hosts (Jeong et al. 2005; Kwak et al. 2005; Choi et al. 2006; Kvitt et al. 2008). Loop-mediated isothermal amplification (LAMP) is a single tube, single temperature technique for the amplification of nucleic acids (Notomi et al. 2000). For RNA targets, a reverse transcription-coupled LAMP procedure is available (Notomi et al. 2000). This technology has been developed to support diagnostic activities in smaller laboratories where there may be limited or no access to expensive equipment such as thermal cyclers. Commercially available detection kits using this technology are available for a variety of pathogens including bacteria and viruses (reviewed in Mori and Notomi 2009). The application of LAMP for the detection of fish and shellfish pathogens is reviewed in Savan et al. (2005). With respect to the iridoviruses, several authors have reported on the developed LAMP-based diagnostics (Caipang et al. 2004; Mao et al. 2008; Zhang et al. 2009). These procedures use simple detection systems that enable the results of the test to be accessed visually by turbidity or through the use of fluorescent substances such as calcein and SYBR green. As LAMP does not require temperature cycling, and detection of the products does not require the use of sophisticated measurement equipment, this procedure shows great promise for general use in fish health laboratories. However, selection of an appropriate target and primer design depends upon good genomic information for the pathogen. As an example, Mao et al. (2008) report on the successful use of LAMP for detection of SGIV in cell lines and tissues from infected fish. This study used a set of specific primers that targeted the ORF-014L of SGIV and provided a comparison of the three methods that can be used for product detection.

Functional Information from Sequence Data

The analysis of the genome of the Singapore grouper iridovirus (SGIV) resulted in the identification of 162 potential open reading frames (ORFs) (Song et al. 2004; Chapter 9 of this book by Wu et al.). It is difficult to predict from sequence data alone whether ORFs are functional genes. In this instance, the availability of the whole genome sequence allowed these authors to use a combination of comparative genomics, RT-PCR, and gel-based proteomics to identify ORFs that were expressed at both the transcript and protein level. Through their proteomics studies, which were labor-intensive, they successfully identified 26 proteins, of which 20 were novel from purified virions grown in embryonic egg cell line from grouper. The identification of these novel proteins allowed them to confirm an additional 12% of the ORFs as functional genes.

To examine in more detail the transcription program of iridoviruses, and to aid in genome annotation, a number of groups have developed DNA microarrays. For example, Lua et al. (2005) developed a red sea bream iridovirus (RSIV) DNA microarray containing 92 putative ORFs that was used to monitor gene transcription, to assign RSIV transcripts to different expression classes, and to study their physical position relative to other genes within the genome. These studies were conducted in vitro using a Japanese flounder (*Paralichthys olivaceus)* natural embryo (HINAE) cells, some of which had been treated with drugs that inhibited protein synthesis and viral DNA replication. They reported that viral replication began after about 8 hours and that it took about 2 days to assemble complete virions in this experimental system. In addition, they were able to confirm that RSIV followed the classical virus gene expression cascade. Using this same array, Lua et al. (2007) examined the transcriptional response of spleen and kidney of juvenile red seabream

(*Pagrus major*) following injection challenge with RSIV. On the basis of the levels of transcription, they suggested that pathogenesis began around 5 days postinfection (DPI) with high levels of viral replication occurring until 10 DPI when clearance of the virus by the host began. On the basis of the transcription profiles, they also suggested that the spleen was the target organ of preference for RSIV. Chen et al. (2006) constructed another microarray containing probes for the 162 SGIV ORFs that had been identified by Song et al. (2004). They were able to identify 127 ORFs that were active upon infection of a grouper (*Epinephelus coioides*) embryonic cell line, as well as to study the timing of their expression. In another study, Teng et al. (2008) reported on the transcriptional response of SGIV following in vitro and in vivo infection using a grouper spleen cell line and the orange-spotted grouper, respectively. The microarray that was used contained all of the SGIV ORFs. These authors noted marked differences in the SGIV transcriptional response between in vitro and in vivo conditions. When grown in cell culture, the transcriptional response was much more rapid with viral genes being transcribed as early as 1-hour postinfection (HPI). Following intraperitoneal injection challenge of grouper, most viral genes were expressed between 1 and 4 DPI with expression levels beginning to decline by 5 DPI. This decline in expression was proposed to be due to the host response to the virus.

Proteomics

As mentioned previously, Song et al. (2004) conducted preliminary proteomic analysis of purified virions of SGIV using a gel-based proteomics approach. Using more advanced proteomic methods (1-DE- MALDI and LC-MALDI), Song et al. (2006) was able to more effectively identify proteins from purified SGIV virions. As in their earlier study, these virions were produced in an embryonic egg cell line from grouper. Using these methods, 44 viral proteins were identified, including 19 proteins that had previously been identified by Song et al. (2004) (see Chapter 9 in this book by Wu et al.). Taken together, these two studies have identified 51 SGIV proteins, which equals 32% of the predicted ORFs as functional genes. Following on this work, Chen et al. (2008) used proteomics to examine changes in the proteomes of SGIV and the grouper embryonic cell line following infection. These authors used isobaric tags for relative and absolute quantification (iTRAQ), which is a nongel-based technique that enables the identification and quantification of proteins from different sources in a single experiment (described in Pierce et al. 2008 and references therein). This is the first fish health application of this labeling system. In this study, noninfected and infected cells harvested at 48 HPI were individually labeled with iTRAQ reagents. After labeling and further processing, the samples were pooled and initially separated by 2D-liquid chromatography with the resulting fractions analyzed in a MALDI TOF/TOF mass spectophotometer. Using these methods, these authors were able to identify 49 viral proteins, including 11 that had not been found previously by Song et al. (2004, 2006). As described below, they also identified 743 host proteins, including several whose abundance changed in response to infection.

Taken together, these studies demonstrate that, even with a relatively simple fully sequenced genome, it is still very difficult to obtain data on the complete proteome, especially for pathogens that must be grown in cell culture. The above statement notwithstanding, these types of studies have greatly improved our understanding of the biology and mechanisms of pathogenesis of iridoviruses, making it possible for the design of experiments to confirm the function of specific transcripts and their products, examples of which are described below.

Bioinformatic analysis of all fully sequenced iridoviruses has identified the presence of an ORF that is a putative RNase III gene. This gene has been shown to be upregulated in iridoviruses in later stages of infection by microarray and proteomic analysis (Lua et al. 2005; Chen et al. 2006; Dang Thi et al. 2007; Chen et al. 2008). In other groups of viruses, this gene has been demonstrated to enhance viral RNA silencing activity in host cells through its interaction with the viral protein P22 (Kreuze et al. 2005). Zenke and Kim (2008) conducted a functional characterization of the putative RNase III of the RBIV. These authors demonstrated the function for this gene by producing recombinant RBIV RNase III that degraded dsRNA. However, the ionic requirements for its function differ from those described for other RNase IIIs. On the basis of the

RT-PCR results, they reported expression of this gene late in the infection process (48 HPI), which is the pattern of expression reported for this gene in other iridoviruses. Although they demonstrated that this gene is functional in RBIV infections, it remains to be determined what, if any, role it plays in viral replication or avoidance of host immune responses.

Viruses have evolved methods to regulate host signaling pathways, thereby ensuring their survival and propagation in host cells. One of these methods involves the modulation of tumor necrosis factor (TNF). Through bioinformatic analysis, Huang et al. (2008) were able to identify a putative lipopolysaccharide-induced TNF-α (LITAF) homolog in the genome of SGIV. Expression patterns of this gene were studied by RT-PCR and Western blotting, which used antibodies raised against recombinant LITAF produced in *Escherichia coli*. This antibody was also used to localize LITAF production in infected cells, where it was found to be associated with mitochondria. To examine the role in pathogenesis, cells were transfected with a LITAF plasmid. Overexpression of LITAF induced apoptosis, resulting in the depolarization of mitochondrial membranes and activation of caspase 3. Using a reported gene system, increased activity of the transcription factors NF-$\kappa\beta$ and NFAT was seen in LITAF-expressing cells. These transcription factors are involved in the regulation of cell cycles, cell differentiation, apoptosis, as well as immune response to virus. Taken together, these data provide evidence that LITAF is important in viral replication, transmission between cells, and possible evasion of the host immune response (Huang et al. 2008).

Morpholinos are a commonly used knockdown system, especially in embryological studies. They are synthetic molecules that act by steric blocking (binding to a target sequence within RNA) and physically interfering with other molecules that would normally interact with the RNA. This system has been used to study gene function in two iridoviruses, SGIV, and ISKNV (Wang et al. 2008a, 2008b). With respect to SGIV, high abundances of the protein encoded by the gene ORF018R were identified in proteomic studies of SGIV, suggesting an important role in infection (Song et al. 2004, 2006; Chen et al. 2008). In addition, bioinformatic analysis of this gene identified a partial serine/threonine kinase domain, suggesting that the protein may be involved in phosphorylation. Wang et al. (2008a) used an antisense morpholino oligonucleotide (asMO) to knock down SGIV ORF018R expression in virus growing in an embryonic grouper cell line. They were able to demonstrate dramatically reduced expression of ORF018R, and corresponding partial inhibition of expression of other SGIV genes normally expressed during late stages of infection, blocking of viral infection, effects on viral DNA packaging, and virion assembly and enhanced phosphorylation (Wang et al. 2008a). Effects on viral and host protein phosphorylation state were confirmed by proteomics analysis (2DE-MS). asMOs have also seen application in studies of iridovirus gene function of other groups of animals. For example, Sample et al. (2007) used asMOs to examine the function of three genes of the frog virus referred to as frog virus 3 (FV3) growing in a fathead minnow (*Pimephales promelas*) cell line. Using asMOs, these authors were able to transiently knockdown the expression of genes believed to be involved in a variety of points in this virus's lifecycle. These included the major capsid protein (MCP), an 18 kDa immediate-early protein (18K) and the FV3 homolog of the largest subunit of RNA polymerase (vPol-IIα). Using transmission electron microscopy to examine virus growth, the authors were able to identify phenotypic changes associated with the knockdown of MCP, as well as the importance of MCP and vPol-IIα in the replication of FV3 in this cell line. Collectively, these studies provided a good demonstration of the value of using asMOs to study the gene function in pathogens.

Wang et al. (2008b) examined the function of infectious spleen and kidney necrosis virus (ISKNV) ORF48R in the infection of zebrafish. Bioinformatic analysis of the ISKNV genome identified a domain similar to that of the platelet-derived growth factor and vascular endothelial growth factor (VEGF) within this ORF, which was similar to VEGFs of other iridoviruses. To understand its role in infection and disease, a recombinant plasmid containing ISKNV ORF48R was constructed and microinjected into one-cell stage zebrafish embryos. The effects of overexpression of ISKNV ORF48R were examined over early embryonic development and were found to be similar to the effects seen when

embryos were infected with a plasmid containing zebrafish (*Danio rerio*) VEGF. These effects included pericardial edema and dilation of the tail region. Overexpression of both constructs with an embryo resulted in phenotypes with similar but much more severe vascular development problems. These authors used a combination of real-time PCR, whole mount in situ hybridization, and immunoprecipitation to identify that the endothelial cell-specific tyrosine kinase receptor (FLK-1) is an important receptor for ISKNV ORF48R. This was further confirmed by knock down of the expression of flk1 with antisense morpholino-oligonucleotides, which when followed by ORF48R over expression initially resulted in a lessening of the severity of the abnormalities.

RNA interference or sequence-specific gene silencing is widely used to examine gene function (gene-knockdown studies) as well as in genetic engineering and medicine. In human medical studies, short interfering RNA (siRNA) has been tested as therapies against a growing number of diseases and pathogens, especially those for which there are limited treatment options. A good review of RNA interference and its medical applications is given in Kim and Rossi (2007). With respect to the iridoviruses, knowledge of the genome enabled Dang Thi et al. (2008) to investigate in vitro the antiviral activity of a siRNA that targeted the MCP gene of RSIV. They demonstrated a dose-dependent effect of this siRNA on RSIV replication. In addition to demonstrating the potential of siRNA as a therapy against RSIV, this study provides an example of how RNA interference can be used to investigate mechanisms of pathogenesis. Although not an example from the Iridoviridae, another recent example on the application of RNA interference in the study of viral pathogenesis of Betanodavirus in fish is provided by Su et al. (2009). Because of the availability of sequence information, these authors were able to use RNA interference, as well as transient expression in a cell line of the viral nonstructural protein B2, to the study of the role of B2 in the pathogenesis of Betanodavirus. With these techniques, they were able to determine that B2 plays an important role in mitochondria-mediated necrotic cell death, possibly through its regulation of the proapoptotic gene Bax.

Bacterial Pathogens—*A. salmonicida*

When compared to the viruses, there are far fewer complete genome sequences for bacterial pathogens of fish (Table 6.2). This is due in part to the larger size and complexity of their genomes; until recently, sequencing their genomes was much too expensive for most research groups. However, with recent developments in sequencing technologies that have dramatically reduced sequencing costs, it is expected that more genome sequences will become available for bacterial pathogens of fish in the near future.

To demonstrate the value of genomic resources as a basis for the study of bacterial pathogens of fish, we will use *A. salmonicida* as an example. *A. salmonicida* is a nonmotile, Gram-negative bacterium that is the etiological agent of furunculosis, an economically important disease of wild and farmed fish. This pathogen is associated with covert and clinical disease in a wide variety of salmonid and nonsalmonid fishes held in fresh and marine waters. The typical form *A. salmonicida* subsp. *salmonicida* has been reported to cause disease in most species of salmonids. In chronic cases, the typical form of the disease is characterized by the presence of furuncle-like swellings that eventually rupture to form ulcerative lesions. Infected fish may display exophtalmia, bloody fins, darkening of skin color and septicemia (Hiney and Olivier 1999). A large number of atypical forms of *A. salmonicida* have been isolated from a large number of nonsalmonid hosts (Wiklund and Dalsgaard 1998). These subspecies, which are heterogenous with respect to molecular and phenotypic characteristics, cause a variety of disease conditions referred to as atypical furunculosis or ulcer disease (Hiney and Olivier 1999).

Because of the economic importance of *A. salmonicida* subsp. *salmonicida* as a disease-causing agent, the whole genome of a wild-type strain (A449) was sequenced and annotated. The genome consists of a circular chromosome and five plasmids encoding a total of more than 4700 genes (Reith et al. 2008). As we will see our knowledge of the genome of *A. salmonicida* and of the related species *Aeromonas hydrophila,* (Seshadri et al. 2006) have enabled subspecies comparisons of the different *A. salmonicida* subspecies, investigations of *A. salmonicida salmonicida* host–pathogen

Table 6.2. Genomic Sequencing of Important Bacterial Pathogens of Fish (data from Entrez Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez).

Pathogen	Disease	Distribution	Status (As of November 2009)	Reference
Gram Negative				
Aeromonas hydrophila	Hemorrhagic septicemia, motile *Aeromonas* septicemia, ulcer disease, red-sore disease	Freshwater to marine	Completed	Seshadri et al. 2006
Aeromonas salmonicida subsp. *salmonicida*	Furunculosis	Freshwater to marine	Completed	Reith et al. 2008
Edwardsiella ictaluri	Enteric septicemia in catfish (ESC)	Freshwater	In progress	
Edwardsiella tarda	*Edwardsiella* septicemia (ES)	Freshwater to marine	In progress	
Flavobacterium psychrophilum	Cold water disease	Freshwater	Completed	Duchaud et al. 2007
Vibrio alginolyticus	Vibriosis	Freshwater to marine	In progress	
Vibrio campbellii	Vibriosis	Brackish to marine	In progress	
Vibrio harveyi	Luminous vibriosis	Marine	Completed	Unpublished sequence in Genbank
Vibrio parahaemolyticus	Vibriosis	Brackish to marine	Completed	Makino et al. 2003
Vibrio splendidus	Vibriosis	Freshwater to marine	Completed	Unpublished sequence in Genbank
Vibrio vulnificus	Vibriosis	Freshwater to marine	Completed	Chen et al. 2003
Nocardia farcinica	Nocardiosis	Freshwater to marine	Completed	Ishikawa et al. 2004
Yersinia ruckeri (ATCC 29473)	Enteric red mouth disease (ERM)	Freshwater to marine	In progress	
Gram Positive				
Lactococcus garvieae	Streptococcosis or lactococcosis	Freshwater to marine	In progress	
Mycobacterium marinum	Mycobacteriosis	Freshwater to marine	Completed	Stinear et al. 2008
Renibacterium salmoninarum ATCC 33209	Bacterial kidney disease (BKD)	Freshwater to marine	Completed	Wiens et al. 2008
Streptococcus iniae	Streptococcosis	Freshwater to marine	In progress	

interactions, and facilitated the development of new vaccines.

Genomic Variability and Subspecies Identification

A variety of molecular analysis such as DNA:DNA hybridization, plasmid profiling, restriction endonuclease fingerprinting, and randomly amplified polymorphic DNA have been used to study the genetics of *A. salmonicida* (reviewed in Nilsson et al. 2006; Nash et al. 2006). In addition to these more general methods, *A. salmonicida*-specific PCR-based methods have also been developed for the identification of strains/subspecies of *A. salmonicida* (Gustafson et al. 1992; Hiney et al. 1992; Miyata et al. 1996; Nilsson et al. 2006; Beaz-Hidalgo et al. 2008). All of these PCR-based methods are based upon the analysis of single genes that limits their usefulness as tools to study genomic variability between subspecies, strains, and isolates.

Burr and Frey (2007) examined 40 isolates of typical and atypical *A. salmonicida* for the presence of five genes involved in type III secretion system by PCR. This is a good example of how genomic PCR amplification can be used as an economical method for the study of small numbers of previously characterized genes. However, it is unsuitable for application to the study of larger numbers of genes that is necessary to fully understand genomic variability within this species.

Genome arrays containing large numbers of genes, or whole genomes in some cases, are the basis for a technique referred to as microarray-based comparative genomic hybridization. This technique has seen application in the study of the genomic basis of diseases and other disorders, as well as the study of genomic variability among strains of bacterial pathogens, including *A. salmonicida* (Nash et al. 2006; Han et al. 2008; Gouré et al. 2009). In the case of *A. salmonicida*, Nash et al. (2006) utilized the genome sequence to develop a 2024 gene array. This microarray was used to study the genetic relationships between species, subspecies, and strains of *Aeromonas* obtained from different hosts and geographical locations. It allowed the authors to identify subsets of conserved genes involved in virulence that are potential vaccine candidates. In addition to identifying conserved genes, some of which may be suitable as targets for the development of new or more effective vaccines, this technique also allows for detailed study of the relationships between groups of bacteria, which has application in epizootiological studies.

The availability of a sequenced genome has also facilitated studies of *A. salmonicida* virulence mechanisms and growth. Prior to the sequencing of the *A. salmonicida* genome, only a few genes encoding for virulence factors, such as the type III secretion system and associated toxins, and quorum sensing had been identified and sequenced (Swift et al. 1997, 1999; Burr et al. 2002, 2003, 2005). Sequencing, bioinformatic analysis, and annotation of the *A. salmonicida* genome resulted in the identification of large numbers of genes encoding for other potential virulence factors, as well as those involved in important biological processes such as growth (Boyd et al. 2003; Reith et al. 2008).

A variety of methods have been used to investigate the mechanisms of virulence in *A. salmonicida*. Low-throughput methods include the construction of mutant strains that lack or overexpress the gene or genes of interest, which are then tested for their virulence using in vitro and/or in vivo models. An example of such an approach is the one used to study the type III secretion system. The type III secretion system consists of an injection system (membrane proteins and a needle-like structure) and a number of effector proteins that are transmitted into host cells, where they modulate components of innate and adaptive immune response. In the case of *A. salmonicida,* the type III secretion system has received the largest amount of study because of its recognized importance as a virulence factor in other bacterial species (Burr et al. 2005; Dacanay et al. 2006). Using techniques such as marker-replacement mutagenesis, these authors were able to demonstrate attenuated virulence following challenge in strains with mutations in genes that made up the structural components of this system. Mutations in individual secreted effectors (aexT, aopH, and aopO) did not result in attenuated virulence, although a reduction in virulence following injection challenge was noted in the aexT, aopH mutants (Dacanay et al. 2006). In contrast, a mutation in the pore-forming gene (ascC), through which these effectors are secreted,

resulted in a lack of virulence following injection and immersion challenge (Dacanay et al. 2006). It is noteworthy that fish challenged with ascC lacked the protective immunity provided to survivors that had been challenged with the wild-type strain. Thus, a combination of genomic analyses, directed mutants, and live challenges can identify not only virulence mechanisms but also candidates for vaccines development.

Pili are structures that allow bacteria to attach to surfaces such as host tissues, and they are important virulence factors for many pathogenic bacteria. Bioinformatic analysis of the *A. salmonicida* subsp. *salmonicida* genome led to the identification of three genes belonging to the type IV pilus system, of which one was thought to be nonfunctional (Boyd et al. 2008). Using this information, three mutant strains were produced and tested for virulence by injection and immersion challenge. These included mutants in genes that encode for components of the Tap pili (tapA), the FLP pili (flpA), and a double (flpA-tapA) mutant. The virulence of the tapA and flpA-tapA mutants was reduced when compared to wild type in immersion challenges but not in injection challenges. There was no difference in virulence between the wild type and the flpA mutant, in both immersion and injection challenges. On the basis of these data, it was concluded that Tap pili are important in initial attachment to the host but not as important to virulence once invasion has occurred.

Host phagocytic cells use the deleterious biological effects of reactive oxygen species as part of their nonspecific host defense against pathogens. Using the genomic sequence of A449, Dacanay et al. (2003) identified two ORFs that code for two superoxide dismutase isozymes, sodA and sodB, examined their in vitro and in vivo patterns of expression, and quantified differences in SOD levels between virulent and avirulent isolates of *A. salmoncida* subsp. *salmonicida*. Using these and genomic data, knockout mutants for genes (sodA and sodB) and a catalase (katA), which is involved in hydrogen peroxide detoxification, were created and used in challenge trials with Atlantic salmon. When challenged by injection, there was no significant change in virulence in any of the mutant strains when compared to wild type. Immersion challenge of the sodA and sodB mutants resulted in reduced virulence when compared to wild type (Boyd and Dacanay, personal communication).

The development of individual mutants, and testing of their phenotypes, is a very effective low-throughput method for studying gene function, especially as it pertains to growth and virulence. However, the availability of a sequenced genome allows for the use of higher throughput methods for the study of gene function. For example, it is now possible to construct complete and ordered mutant libraries for bacteria and to use genomic DNA arrays or other methods to screen for negatively selected transposon mutants (reviewed in Baldwin and Salama 2007; Burrack and Higgins 2007; Winterberg and Reznikoff 2007). The use of these techniques has been recently applied to study virulence genes of the bacterial fish pathogens *Mycobacterium marinum* (Mehta et al. 2006), *Lactococcus garvieae* (Menéndez et al. 2007), and *Edwardsiella ictaluri* (Karsi et al. 2009). In these studies, the ability of mutants to survive and/or replicate in the presence of host factors and cells under in vitro and in vivo conditions was used as a screen to identify virulence-related genes. These studies enabled the authors to identify a large number of virulence-related genes and, in the case of *E. ictaluri*, to identify mutants that have potential as live attenuated vaccines.

Proteomics

Proteomics techniques have been used to survey proteins produced by *A. salmonicida* and related species under a variety of different growth conditions, as well as to delineate structural details of small numbers of specific proteins. Most protein surveys have used gel-based proteomics methods, whereas structural studies of single or small groups of proteins have used gel-free proteomic methods such gas–liquid chromatography–mass spectrometry. Most proteomic studies on *A. salmonicida* have focused on membrane and membrane-associated proteins because of the roles they play in pathogenesis, such as adhesion to host cells, antibiotic, and drug resistance, as well as their potential as vaccine candidates.

A number of groups have conducted general surveys of secreted and membrane-bound proteins of *A. salmonicida*. Two-dimensional PAGE-based proteomics was used to compare the outer membrane

proteins (OMP) of *A. salmonicida* grown in vitro in standard microbiological and low-iron media, as well as in vitro (within dialysis tubing surgically implanted into hosts) (Ebanks et al. 2004, 2005). Ebanks et al. (2004) examined the OMP of wild type and two avirulent strains (one A-layer positive and one A-layer negative) in response to low iron in vitro and in vivo growth. Under these conditions, two iron acquisition systems were found to be upregulated as represented by three proteins in the 73–85 kDa size range in all of these strains. The availability of a sequenced genome of *A. salmonicida* allowed for these authors to identify these proteins as a ferric siderophore receptor homolog (FstB; 85 kDa), a colicin receptor homolog (FstC; 73 kDA), and an outer membrane heme receptor homolog (76 kDa). Microarray analysis of mRNA expressed in strain A449 grown under the same conditions confirmed the results obtained in the proteomics experiments described above (Brown et al., unpublished). Although important for growth in iron-poor environments, these proteins were not felt to be sufficient for bacterial virulence as they were expressed in both virulent and avirulent strains (Ebanks et al. 2004). Further study of the OMP of *A. salmonicida* grown under iron-limited conditions was reported in Ebanks et al. (2005). In this study, wild-type *A. salmonicida* (strain A449) and an avirulent s-layer-deficient mutant were used, and OMPs were extracted using a modified method designed to enrich for OMPs. These authors were able to identify 76 proteins, which corresponded to approximately 60% of all of the protein spots that could be visualized by on their gels. Within OMPs, a number of proteins were identified, which, due to their lack of a classical export sorting signals, had not been predicted during sequence analysis of the genome to be OMPs. This result demonstrates the value of combining genomic and proteomic approaches in such studies. Another example of a combined genomics and proteomics approach is demonstrated by studies of the type III secretion system. As previously mentioned, the availability of genomic information for *A. salmonicida* has allowed for the development of a number of mutant strains that included mutations within the type III secretion system. Ebanks et al. (2006) used 1D and 2D gel-based proteomics and reverse transcriptase (RT)-PCR to study the type III secretion system of a wild type (A449) and two mutant strains of *A. salmonicida* grown in vivo, as well as under a variety of in vitro growth conditions. Using differential proteomic analysis and RT-PCR, expression of genes and proteins of the TTSS was shown to be upregulated at a growth temperature of 28°C, but not at lower temperatures or in cells cultured in low iron, low pH, low calcium, or in vivo. In this study, an ascC deletion mutant with a nonfunctional TTSS was used as a control in the proteomic identification of TTSS and other proteins produced during growth at 28°C.

More recently, several authors have used a combination of sequence information and proteomics to study the genes involved in LPS core structures in selected strains of *A. salmonicida* (A450) and to compare them to those of *A. hydrophila* (Jimenez et al. 2009). Within one of the three genomic regions studied, these authors identified seven LPS core biosynthesis genes, of which three were identical in sequence to those of *A. hydrophila*. Mutants were constructed with alterations to the four nonidentical genes, and their LPS core structures were characterized. Using these data along with complementation studies to rescue LPS production in *A. hyrdophila* LPS core biosynthesis mutants, and SDS-PAGE analysis of LPS, allowed these authors to assigned function to all of the genes involved in *A. salmonicida* A450 LPS core biosynthesis.

A combined microarray and proteomics study was used to examine changes in transcription and translation levels for *A. salmonicida* in response to iron-restricted in vitro and in vivo growth conditions (Brown et al., unpublished). Comparison of gene expression data to proteomic data obtained from the same samples showed overall that there was a strong positive correlation between gene and protein expression levels. Not surprisingly, many of the genes that were differentially expressed were involved in iron or heme utilization. However, there were several genes that were only upregulated when bacteria were grown in the host and these included genes homologous to an ABC-type galactoside transporter and components of the lateral flagellar system.

HOST–PATHOGEN INTERACTIONS

There are four recent reviews that provide excellent detail on genomic tools and their application to

the study of immune gene function in fish (Douglas 2006; Dios et al. 2008; Goetz and MacKenzie 2008; Martin et al. 2008). With the exception of a few model fish species (e.g., zebrafish, pufferfish), for which there are sequenced genomes, the identification of genes and pathways involved in fish response to pathogens has generally relied upon EST sequencing of cDNA libraries from immune-related tissues and associated bioinformatics analysis. Many early studies used nonnormalized cDNA libraries, which resulted in relatively low rates of immune gene discovery. Recently, normalized cDNA and especially suppression subtractive hybridization (SSH) cDNA libraries have been widely used to improve rates of immune gene discovery and in the case of SSH libraries to enrich for genes whose expression changes in response to antigen/antigens or disease challenge. The resulting cDNA clones and sequence information have been used to develop PCR-based assays, cDNA microarrays, and oligonucleotide microarrays.

The majority of fish health studies conducted to date examined host response by studying single or small subsets of genes using PCR-based methods (RT-PCR and real-time PCR) (reviewed in Dios et al. 2008). A smaller but rapidly growing number of studies are using microarrays for transcriptional profiling of host responses to infection. To date, the majority of studies have used cDNA microarrays that were generated by the same research teams, from clones produced during their studies. There are few publically available microarrays for fish (Agilent 4 × 44 K zebrafish and Atlantic salmon microarray platforms are available), and for nonmodel fish species, salmonid cDNA microarrays produced by cGRASP are readily accessible. Since their release, these arrays, which are suitable for use with most salmonid species, have seen use in over 22 studies, including 7 fish health-related studies (reviewed in von Schalburg et al. 2008). Recently, several groups have reported on the development of oligo-based microarrays for species suh as Atlantic salmon, turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), and Atlantic cod (*Gadus morhua*), which are designed to support studies of immune function (Cerdà et al. 2008; von Schalburg et al. 2008; Millán et al. 2009; Booman et al., 2011; Booman and Rise 2011, Chapter 1 of this book). Oligo-based microarray development for fish has been facilitated by ever-increasing availability of sequence information and the availability of commercial companies that can produce high-quality arrays in a timely and cost-effective manner.

With respect to the Iridoviridae, studies on their interactions with their hosts are very limited. In rockbream (*Oplegnathus fasciatus*), transcriptional responses of isoforms of hepcidin and glyceraldehyde 3-phosphate dehydrogenase have been identified following challenge with RBIV by real-time PCR (Cho et al. 2008, 2009). Using SSH library construction and RT-PCR, differential gene expression was studied in the spleen of mandarin fish (*Siniperca chuatsi*) challenged with ISKNV (He et al. 2006). Although this was a modest study that sequenced only 386 ESTs, it still provides good insights into the genes and gene pathways that make up the response of this species to ISKNV.

In the case of *A. salmonicida,* host responses were used to investigate aspects of both pathogen and host biology. Fast et al. (2009) provides an example of a targeted study that utilized mutant strains of *A. salmonicida* to examine pathways of macrophage activation in Atlantic salmon. In their study, deletion mutants of the type III secretion system of *A. salmonicida* subsp. *salmonicida* were used to study the responses of Atlantic salmon anterior head kidney leucocytes to infection (Fast et al. 2009). These mutants included a strain with a deletion in the outer membrane pore gene (ascC) and a triple effector knockout strain (Δaop3) formed by deletion of effector genes aopO, aopH, and aexT. As mentioned previously, the ascC mutant is avirulent and not protective with respect to subsequent challenge with wild-type *A. salmonicida*. Their results support the view that the type III secretion system is important with respect to survival within host cells through polarization of macrophages/leucocytes to an alternative, rather than classical, activation state. With respect to the ascC mutant, its short-term survival within host cells and lack of T-cell signaling cytokine stimulation may explain why hosts exposed to this mutant are not protected to subsequent wild-type challenge (Fast et al. 2009).

Researchers studying *A. salmonicida* were the first to apply cDNA microarray technology to study the response of fish to pathogen challenge

(Tsoi et al. 2003). At the time of their study, there were no microarrays available for fish. Therefore, these researchers used a commercially available human cDNA microarray to identify differentially expressed genes in the liver of Atlantic salmon challenged with *A. salmonicida*. This study had limited success in identifying differentially expressed genes because of the high levels of sequence divergence between Atlantic salmon and humans.

Building upon this earlier work, six SSH cDNA libraries were constructed for liver, head kidney, and spleen tissues following i.p. challenge with *A. salmonicida* (Tsoi et al. 2004). From these libraries, 1778 clones were selected and used to create a custom cDNA microarray, which was used to study the transcriptional responses of Atlantic salmon following cohabitation challenge with *A. salmonicida* and Atlantic salmon macrophages exposed in vitro to in vivo and in vitro cultured *A. salmonicida* (Ewart et al. 2005, 2008). These studies have provided insights into the early (<2 hours postinfection) and late (chronic disease, 13 days postinfection) transcription response of Atlantic salmon to this pathogen and have identified a number of unknown genes that are differentially regulated and worthy of future study. In addition, Ewart et al. (2008) provided evidence for differences in the transcriptional response of macrophages to bacteria grown in vivo and in vitro. These studies demonstrate the value of microarray technology and the value of targeting of research in this area to examine in more detail the effects of specific *A. salmonicida* proteins or other products on the host.

As mentioned earlier, the availability of Atlantic salmon microarrays from cGRASP has made it possible for a number of research groups to examine the transcriptional response to pathogens. Martin et al. (2006) used real-time PCR, SSH library construction, and an early version of the cGRASP microarray to examine the response of Atlantic salmon gill, kidney, and liver tissues to challenge with an attenuated strain of *A. salmonicida*. This strain has a mutation in its aeroA gene that results in it being rapidly cleared from the host after only a short period of replication without morbidity in the host. In addition, vaccination with this mutant is known to provide a protective immune response. In this study, microarray analysis and real-time PCR was used to confirm the results obtained from analysis of the SSH libraries, as well as to identify additional genes not identified in these libraries but whose expression changed in response to challenge. Included in these genes were many members of acute phase response pathways. This type of study greatly improves our understanding of how the immune system responds to bacterial challenge, the impact of challenge on nonimmune-related pathways and the mechanisms by which resistance develops. These insights are important with respect to the development of new thereaputants and vaccines for fish.

More recently, Pardo et al. (2008) reported on 9256 ESTs (3482 unique sequences) from turbot (*S. maximus*) that had been challenged with *A. salmonicida salmonicida* and the ectoparasitic protozoan *Philasterides dicentrarchi*, as well as from unchallenged individuals. On the basis of the analysis of EST frequency, using a subset of 72 genes, they were able to identify differences in the frequency of a number of genes between animals challenged with these two pathogens. Using these ESTs data, a low-density 2716 probe oligo-based Agilent microarray was recently developed to study the response of turbot to pathogens (Millán et al. 2009). These authors note that this microarray is publically available on a cost-recovery basis. Testing of this microarray using spleen tissue from *A. salmonicida*-challenged fish demonstrated its usefulness as a tool to support studies on and a broodstock selection program for this commercially important fish.

Using a microarray developed by the EU Framework 5 GENIPOL consortium for toxicogenomics studies of the European flounder, Diab et al. (2008) examined the transcriptional response of liver of European flounder injected with a commercial *A. salmonicida* vaccine over a 16-day period. Although this microarray was originally designed to study liver responses of flatfish to toxicants, there were sufficient immune-related genes present to allow these authors to obtain good information for a number of immune-related groups such as antimicrobial proteins, acute phase proteins, complement system, chemokines, and cytokines. This study demonstrated that this array is suitable to begin to examine the effects of exposure to toxicants on the immune system of this and other species of related

flatfish. These authors also note that this array is available to the public for research purposes.

A similar strategy was used for immune-related gene discovery in Atlantic cod (*G. morhua*) that were stimulated with formalin-killed atypical *A. salmonicida* (Feng et al. 2009). In their study, four reciprocal SSH cDNA libraries were created to identify differentially expressed genes in spleen and head kidney tissues. From analysis of the 4154 ESTs and real-time-PCR of a select subset of genes, they were able to identify a relatively large number of genes involved in many biological processes, including chemotaxis, regulation of apoptosis, antimicrobial peptide production, and iron homeostasis that responded to bacterial antigen stimulation. These EST data, along with additional ESTs from other SSH and normalized cDNA libraries, have been recently used to produce a 20,000 element oligo-based microarray that is designed to support research on cod reproduction, physiology, and immunology (Booman et al., 2011; Booman and Rise, Chapter 1).

Host Proteomics

As mentioned previously, Martyniuk and Denslow (2009) provide a good overview of proteomic methods focusing on their application to the study of fish endocrinology. With respect to fish health, proteomics has seen limited use in the study of hosts with the few studies that have been conducted being limited to cell lines. Martin et al. (2007) examined the effects of administration of recombinant IFN-γ on the Atlantic salmon head kidney cell line, SHK-1, protein expression. Using 2D gel electrophoresis, these authors created proteome maps for unstimulated and stimulated cells harvested at 24 hours post stimulation. Analysis of these maps resulted in the identification of 15 proteins that increased and 7 proteins that decreased in abundance. Sequencing of some of these proteins and real-time PCR studies of their gene expression supported the results obtained from their proteomics data.

As mentioned above, Chen et al. (2008) used a nongel-based method involving isobaric labeling (ITRAC®) to compare and quantify in the proteomes of grouper embryonic cell line that were uninfected and infected with SGIV. In addition to identifying 49 viral proteins, they also identified 743 host proteins, of which 14 were upregulated and 5 were downregulated in the infected cells (Chen et al. 2008). Host proteins that were upregulated included those involved in immunity, as well as proteins thought to be necessary to support viral replication.

Host Metabolomics

Metabolomics has been applied to characterize small molecular weight metabolites in plasma and tissue extracts of fish exposed to toxicants, other environmental stressors, and pathogens (Viant et al. 2003, 2005; Solanky et al. 2005; Stentiford et al. 2005; Dacanay et al. 2006; Samuelsson et al. 2006; reviewed in Samuelsson and Larsson 2008; Karakach et al. 2009). With respect to pathogen exposure, metabolomic experiments are limited to a series of related studies on Atlantic salmon and *A. salmonicida* that were conducted within the same program as the genome sequencing. As part of this program, Solanky et al. (2005) used NMR-based metabolomics to study and compare the metabolite profiles of plasma obtained from fish that had survived a challenge with virulent *A. salmonicida*, to saline-injected and unfed control groups. These authors were able to identify distinct NMR-spectra (metabolite profiles) for each of these groups, as well as to characterize the major metabolite changes responsible for the differences. This study demonstrated that it is feasible to use metabolomics to develop improved understanding of the relationships between *A. salmonicida* and Atlantic salmon, as well as a method for the identification of infected and noninfected individuals. Dacanay et al. (2006) used ^{1}H NMR spectroscopy-based metabolite profiling to compare plasma metabolite profiles of Atlantic salmon survivors of immersion challenge with four strains of type III secretion system isogenic mutants as well as survivors following rechallenge with wild-type *A. salmonicida*. The animals that were rechallenged were those that had survived the earlier immersion challenge with wild-type *A. salmonicida* or the ΔascC mutant. In that study, a large proportion of the survivors of the wild-type challenge survived the rechallenge. The metabolic response of these animals was distinct from those obtained for a phosphate-buffered saline (PBS) control group and appeared to be related to the protective immune response. With respect to immersion challenge with

the other mutants, there were no apparent differences in their metabolic response between these groups or when they were compared to PBS control animals. This study clearly demonstrates that metabolomics technologies have potential to provide noninvasive assays to monitor host immune responses.

More recently, ^{1}H NMR spectroscopy and ultrahigh performance liquid chromatography–mass spectrometry was used to characterize the response of juvenile Atlantic salmon to long-term handling stress (Karakach et al. 2009). Although not directly related to disease, this paper demonstrates the advantage of using both of these techniques in a complementary fashion for the analysis of low-molecular weight metabolites, outlines the difficulties encountered, as well as addresses some important issues such as the stability of plasma for metabolomics experiments. By comparing plasma from nonstressed controls to that of stressed individuals, the authors demonstrated classifiable differences (metabolic disparity) in metabolite profiles at 1 and 2 weeks after the initiation of the stress. Of note is the fact that such differences were not identifiable at 3 and 4 weeks poststress. The metabolites whose concentration changed in response to stress included lactate, lipoproteins, amino acids, and trimethylamine-*N*-oxide, and their patterns of change suggested changes in gluconeogenessis over time. Furthermore, these metabolite changes revealed several new molecular indicators of long-term stress that had not been previously known for fish.

The studies outlined above represent the first attempts to apply metabolomics to the study the health of aquatic animals. On the basis of these initial studies, it is easy to see how continued application of metabolomics in aquatic animal health and other areas of fish research will facilitate our improved understanding of the interactions between pathogens and their fish hosts, assist in the diagnosis or identification of disease, as well our understanding of factors such as stress that may predispose animals to disease. With respect to the development of "health biomarkers" for fish, Samuelsson and Larsson (2008) bring up several important points to consider with respect to their development and application. For example, they question whether it is possible to determine what a small change in the metabolome of fish that live in a complex environmental setting means with respect to their health. On the basis of their analysis of studies completed to date, they suggest that it may be necessary to use complementary data for robust and accurate classification of disease states. If this is the case, it will likely be necessary to use a combination of biomarkers rather than a single biomarker to indicate a specific state.

APPLICATIONS OF GENOMICS AND PROTEOMICS TO VACCINE DEVELOPMENT

It is generally viewed that development of new and more effective vaccines for fish will benefit the aquaculture industry by further reducing losses due to disease, reducing use of therapeutants (antibiotics and chemical treatments), and promoting improvements to animal welfare. At present, most of commercially available vaccines for fish are inactivated whole cell vaccines, although there is increasing interest and use of subunit vaccines, especially for virus. Vaccines, especially those against bacterial pathogens, generally work well with adjuvanted injectable vaccines giving the best results. However, there are problems with the use of these vaccines, including reduced growth, and the development of adhesions and other pathologies following vaccination that can affect carcass quality (Midtlyng and Lillehaug 1998; Mutoloki et al. 2004; Treasurer and Cox 2008; Gjerde et al. 2009 and references therein).

It is generally agreed that the development of improved vaccines and/or vaccine delivery systems for fish is extremely important, and these goals have been the major rationale for conducting many of the studies reviewed in this chapter. As we have seen, the application of genomics and proteomics to the study of fish pathogens has provided researchers with insights into their biology, their pathogenesis, as well as provided information on potential of vaccine targets. In addition, the application of these technologies to study host response has led to a rapid increase in the number of immune-related genes known for a variety of commercially important fish species. However, it is difficult to determine the extent to which published data have contributed to the development of commercial vaccines. This is due

in part to the fact that most research that has led to the commercialization of vaccines for fish has been performed by pharmaceutical companies and is not available publically.

As discussed previously, the identification and sequencing of virulence factors in *A. salmonicida salmonicida* has enabled researchers to develop a number of mutant strains used to study virulence, as well as, in the case of avirulent strains, to test for use as attenuated vaccines. Although there has been a great deal of interest in the use of attenuated strains as vaccines in aquaculture, and success has been achieved with respect to their development, for example BrivaxII (Marsden et al. 1996, 1998), there are no attenuated vaccines that have been licensed.

In a recent review, Secombes (2008) discussed the importance of immune gene discovery in fish to the rational design and development of new vaccines and adjuvant systems for fish. He noted that a major issue faced in the development of new vaccines and adjuvants is the high costs associated with their development, production, and licensing when compared to simple inactivated pathogen preparations. He continued by suggesting that the genetic adjuvants and vaccines should be considered as a way forward, citing successful licensing and commercialization of a DNA vaccine for fish.

Genetic (DNA) vaccines expressing genes for pathogen proteins are a relatively new approach to vaccine development that is being used in fish, especially in the development of viral vaccines (see Leong et al., Chapter 10 in this book). The advantages of DNA vaccines over autogenous vaccines and their application in aquaculture are described in detail by Kurath (2008). With respect to iridoviruses, Caipang et al. (2006), using the genomic information for RSIV, developed DNA vaccines that expressed a MCP and an ORF containing a transmembrane domain of RSIV. These vaccines were shown to provide effective protection, although the level of protection was not as good as that obtained from a formalin-inactivated RSIV. The availability of genomic information for other viruses has enabled the development and testing of 15 other viral DNA vaccines for fish (reviewed in Kurath 2008). These include the DNA vaccine Apex-IHN for salmon, which is the first licensed DNA vaccine for use in aquaculture.

CONCLUDING REMARKS

In the last few years, there has been a tremendous increase in publications reporting on the application of genomics, proteomics, metabolomics, and associated technologies in fish health research. These technologies have been applied to the studies of pathogen and host biology, interactions between pathogens and hosts, as well as seen application in areas such as vaccine development. These technologies do have some shortcomings that need to be overcome, but overall, they form a tremendously valuable tool set for fish health research.

The application of these technologies has brought about changes in the fish health research community where the traditional laboratory model is giving way to the development of multidisciplinary research teams. Multidisciplinary research teams are key to successful application of these technologies and even more important in programs where these genomics technologies are combined. The development of multidisciplinary teams is necessary to plan, conduct, analyze, and to make biological sense of the large amounts of information generated by these fields of study.

REFERENCES

Baldwin DN and Salama NR. 2007. Using genomic microarrays to study insertional/transposon mutant libraries. Methods Enzymol. 421: 90–110.

Beaz-Hidalgo R, Magi GE, Balboa S, Barja JL, and Romalde JL. 2008. Development of a PCR protocol for the identification of *Aeromonas salmonicida* in fish by amplification of the fstA (ferric siderophore receptor) gene. Vet Microbiol. 128(3–4): 386–394.

Booman M, Borza T, Feng CY, Hori TS, Higgins B, Culf A, Léger D, Chute IC, Belkaid A, Rise M, Gamperl AK, Hubert S, Kimball J, Ouellette RJ, Johnson SC, Bowman S, and Rise ML. 2011. Development and experimental validation of a 20K Atlantic cod (*Gadus morhua*) oligonucleotide microarry based on a collection of over 150,000 ESTs. *Mar Biotechnol*. 13 (4): 733–750.

Boyd J, Williams J, Curtis B, Kozera C, Singh R, and Reith M. 2003. Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. Plasmid. 50(2): 131–144.

Boyd JM, Dacanay A, Knickle LC, Touhami A, Brown LL, Jericho MH, Johnson SC, and Reith M. 2008. Contribution of type IV pili to the virulence of

Aeromonas salmonicida subsp. *salmonicida* in Atlantic salmon (*Salmo salar* L.). Infect Immun. 76(4): 1445–1455.

Burrack LS and Higgins DE. 2007. Genomic approaches to understanding bacterial virulence. Curr Opin Microbiol. 10(1): 4–9.

Burr SE and Frey J. 2007. Analysis of type III effector genes in typical and atypical *Aeromonas salmonicida*. J Fish Dis. 30(11): 711–714.

Burr SE, Stuber K, Wahli T, and Frey J. 2002. Evidence for a type III secretion system in *Aeromonas salmonicida* subsp. *salmonicida*. J Bacteriol. 184(21): 5966–5970.

Burr SE, Stuber K, and Frey J. 2003. The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. *salmonicida* is translocated via a type III secretion pathway. J Bacteriol. 185(22): 6583–6591.

Burr SE, Pugovkin D, Wahli T, Segner H, and Frey J. 2005. Attenuated virulence of an *Aeromonas salmonicida* subsp. *salmonicida* type III secretion mutant in a rainbow trout model. Microbiol. 151(6): 2111–2118.

Caipang CMA, Haraguchi I, Ohira T, Hirono I, and Aoki T. 2004. Rapid detection of a fish iridovirus using loop-mediated isothermal amplification (LAMP). J Virol Methods. 121(2): 155–161.

Caipang CMA, Takano T, Hirono I, and Aoki T. 2006. Genetic vaccines protect red seabream, *Pagrus major*, upon challenge with red seabream iridovirus (RSIV). Fish Shellfish Immun. 21(2): 130–138.

Cerdà J, Mercadé J, Lozano JJ, Manchado M, Tingaud-Sequeira A, Astola A, Infante C, Halm S, Viñas J, Castellana B, Asensio E, Cañavate P, Martínez-Rodríguez G, Piferrer F, Planas JV, Prat F, Yúfera M, Durany O, Subirada F, Rosell E, and Maes T. 2008. Genomic resources for a commercial flatfish, the Senegalese sole (*Solea senegalensis*): EST sequencing, oligo microarray design, and development of the Soleamold bioinformatic platform. BMC Genomics. 9: 508.

Chen C-Y, Wu K-M, Chang Y-C, Chang C-H, Tsai H-C, Liao T-L, Liu Y-M, Chen H-J, Shen AB-T, Li J-C, Su T-L, Shao C-P, Lee C-T, Hor L-I, and Tsai S-F. 2003. Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Genome Res. 13(12): 2577–2587.

Chen LI, Wang F, Song W, and Hew CL. 2006. Temporal and differential gene expression of Singapore grouper iridovirus. J Gen Virol. 87(10): 2907–2915.

Chen LI, Tran BN, Lin Q, Lim TK, Wang F, and Hew CL. 2008. iTRAQ analysis of Singapore grouper iridovirus infection in a grouper embryonic cell line. J Gen Virol. 89(11): 2869–2876.

Choi SK, Kwon SR, Nam YK, Kim SK, and Kim KH. 2006. Organ distribution of red sea bream iridovirus (RSIV) DNA in asymptomatic yearling and fingerling rock bream (*Oplegnathus fasciatus*) and effects of water temperature on transition of RSIZ into acute phase. Aquaculture. 256(1–4): 23–26.

Cho YS, Lee SY, Kim KH, and Nam YK. 2008. Differential modulations of two glyceraldehyde 3-phosphate dehydrogenase mRNAs in response to bacterial and viral challenges in a marine teleost *Oplegnathus fasciatus* (Perciformes). Fish Shellfish Immun. 25(5): 472–476.

Cho YS, Lee SY, Kim KH, Kim SK, Kim DS, and Nam YK. 2009. Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin isoforms resulting from different biological stimulations. Dev Comp Immun. 33(1): 46–58.

Dacanay A, Johnson SC, Bjornsdottir R, Reith M, Ross NW, Singh R, and Brown LL. 2003. Molecular cloning and quantitative analysis of iron and manganese co-factored superoxide dismutases in *Aeromonas salmonicida* subsp. *salmonicida*: re-evaluation of their role in virulence. J Bacteriol. 185(15): 4336–4344.

Dacanay A, Knickle L, Solanky KS, Boyd JM, Walter JA, Brown LL, Johnson SC, and Reith M. 2006. Contribution of the type three secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. Microbiology. 152(6): 1847–1856.

Dang Thi L, Yasuike M, Hirono I, Kondo H, and Aoki T. 2007. Transcriptional profile of red seabream iridovirus in a fish model as revealed by viral DNA microarrays. Virus Genes. 35(2): 449–461.

Dang Thi L, Kondo H, Aoki T, and Hirono I. 2008. Engineered virus-encoded pre-microRNA (pre-miRNA) induces sequence-specific antiviral response in addition to nonspecific immunity in a fish cell line: convergence of RNAi-related pathways and IFN-related pathways in antiviral response. Antivir Res. 80(3): 316–323.

Diab AM, Williams TD, Sabine VS, Chipman JK, and George SG. 2008. The GENIPOL European flounder *Platichthys flesus* L. toxicogenomics microarray: application for investigation of the response to furunculosis vaccination. J Fish Biol. 72(9): 2154–2169.

Dios S, Novoa B, Buonocore F, Scapigliati G, and Figueras A. 2008. Genomic resources for immunology and disease of salmonid and non-salmonid fish. Rev Fish Sci. 16(Suppl 1): 117–130.

Do JW, Moon CH, Kim HJ, Ko MS, Kim SB, Son JH, Kim JS, An EJ, Kim MK, Lee SK, Han MS, Cha SJ, Park MS, Park MA, Lee JS, Kim YC, Choi DL, Kim JW, and Park JW. 2004. Complete genomic DNA sequence of rock bream iridovirus. Virology. 325(2): 351–363.

Do JW, Cha SJ, Kim JS, An EJ, Park MS, Kim JW, Kim YC, Park MA, and Park JW. 2005. Sequence variation in the gene encoding the major capsid protein of Korean fish iridoviruses. Arch Virol. 150(2): 351–359.

Douglas SE. 2006. Microarray studies of gene expression in fish. Omics. 10(4): 474–489.

Duchaud E, Boussaha M, Loux V, Bernardet JF, Michel C, Kerouault B, Mondot S, Nicolas P, Bossy R, Caron C, Bessières P, Gibrat JF, Claverol S, Dumetz F, Le Hénaff M, and Benmansour A. 2007. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nat Biotechnol. 25(7): 763–769.

Eaton HE, Metcalf J, Penny E, Tcherepanov V, Upton C, and Brunetti CR. 2007. Comparative genomic analysis of the familiy Iridoviridae: re-annotating and defining the core set of iridovirus genes. Virol J. 4: 11.

Ebanks RO, Dacanay A, Goguen M, Pinto DM, and Ross NW. 2004. Differential proteomic analysis of *Aeromonas salmonicida* outer membrane proteins in response to low iron and in vivo growth conditions. Proteomics. 4(4): 1074–1085.

Ebanks RO, Goguen M, McKinnon S, Pinto DM, and Ross NW. 2005. Identification of the major outer membrane proteins of *Aeromonas salmonicida*. Dis Aquat Org. 68(1): 29–38.

Ebanks RO, Knickle LC, Goguen M, Boyd JM, Pinto DM, Reith M, and Ross NW. 2006. Expression of and secretion through the *Aeromonas salmonicida* type III secretion system. Microbiol. 152(5): 1275–1286.

Ewart KV, Belanger JC, Williams J, Karakach T, Penny S, Tsoi SCM, Richards RC, and Douglas SE. 2005. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Dev Comp Immunol. 29(4): 33–347.

Ewart KV, Williams J, Richards RC, Gallant JW, Melville K, and Douglas SE. 2008. The early response of Atlantic salmon (*Salmo salar*) macrophages exposed in vitro to *Aeromonas salmonicida* cultured in broth and in fish. Dev Comp Immunol. 32(4): 380–390.

Fast MD, Tse B, Boyd JM, and Johnson SC. 2009. Mutations in the *Aeromonas salmonicida* subsp. *salmonicida* type III secretion system affect Atlantic salmon leucocyte activation and downstream immune responses. Fish Shellfish Immunol. 27(6): 721–728.

Feng CY, Johnson SC, Hori TS, Rise M, Hall JR, Gamperl AK, Hubert S, Kimball J, Bowman S, and Rise ML. 2009. Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*. Physiol Genomics. 37(3): 149–163.

Gjerde B, Evensen Ø, Bentsen HB, and Storset A. 2009. Genetic (co)variation of vaccine injuries and innate resistance to furunculosis (*Aeromonas salmonicida*) and infectious salmon anaemia (ISA) in Atlantic salmon (*Salmo salar*). Aquaculture. 287(1–2): 52–58.

Go J, Lancaster M, Deece K, Dhungyel O, and Whittington R. 2006. The molecular epidemiology of iridovirus in Murray cod (*Maccullochella peelii peelii*) and dwarf gourami (*Colisa lalia*) from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. Mol Cell Probe. 20(3–4): 212–222.

Goetz FW and MacKenzie S. 2008. Functional genomics with microarrays in fish biology and fisheries. Fish Fish. 9(4): 378–395.

Gouré J, Findlay WA, Deslandes V, Bouevitch A, Foote SJ, MacInnes JI, Coulton JW, Nash JHE, and Jacques M. 2009. Microarray-based comparative genomic profiling of reference strains and selected Canadian field isolates of *Actionbacillus pleuropneumoniae*. BMC Genomics. 10: 88.

Gustafson CE, Thomas CJ, and Trust TJ. 1992. Detection of *Aeromonas salmonicida* from fish using polymerase chain reaction amplification of the virulence surface array protein gene. Appl Environ Microb. 58(12): 3816–3825.

Han H, Wong HC, Kan B, Guo Z, Zeng X, Yin S, Liu X, Yang R, and Zhou D. 2008. Genome plasticity of *Vibrio parahaemolyticus*: microevolution of the "pandemic group." BMC Genomics. 9: 570.

He JG, Deng M, Weng SP, Li Z, Zhou SY, Long QX, Wang XZ, and Chan SM. 2001. Complete genome analysis of the mandarin fish infectious spleen and kidney necrosis iridovirus. Virology. 291(1): 126–139.

He W, Yin Z-X, Li Y, Huo W-L, Guan H-J, Weng S-P, Chan S-M, and He J-G. 2006. Differential gene expression profile in spleen of mandarin fish *Siniperca*

chuatsi infected with ISKNV, derived from suppression subtractive hybridization. Dis Aquat Organ. 73(2): 113–122.

Hiney M, Dawson MT, Heery DM, Simth PR, Gannon F, and Powell R. 1992. DNA probe for *Aeromonas salmonicida*. Appl Environ Microb. 58(3): 1039–1042.

Hiney M and Olivier G. 1999. Furunculosis (Aeromonas salmonicida). In: Fish Diseases and Disorders III: Viral, Bacterial and Fungal Infections (ed. Woo PTK and Bruno DW.). CAB Publishing, Oxon, England. pp 341–425.

Huang X, Huang Y, Gong J, Yan Y, and Qin Q. 2008. Identification and characterization of a putative lipopolysaccharide-induced TNF-α (LITAF) homolog from Singapore grouper iriodvirus. Biochem Biophy Res Commun. 373(1): 140–145.

Ishikawa J, Yamashita A, Mikami Y, Hoshino Y, Kurita H, Hotta K, Shiba T, and Hattori M. 2004. The complete genomic sequence of *Nocardia farcinica* IFM 10152. Proc Natl Acad Sci USA. 101(41): 14925–14930.

Jeong JB, Jun LJ, Park HK, Kim KH, Chung J-K, Komisar JL, and Jeong HD. 2005. Asymptomatic iridovirus infection in various marine fishes detected by a 2-step PCR method. Aquaculture. 255(1–4): 30–38.

Jimenez N, Lacasta A, Vilches S, Reyes M, Vazquez J, Aquillini E, Merino S, Regué M, and Tomás JM. 2009. Genetics and proteomics of *Aeromonas salmonicida* lipopolysaccharide core biosynthesis. J Bacteriol. 191(7): 2228–2236.

Karakach TK, Huenupi EC, Soo EC, Walter JA, and Afonso, LOB. 2009. 1H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress. Metabolomics. 5(1): 123–137.

Karsi A, Gülsoy N, Corb E, Dumpala PR, and Lawrence ML. 2009. High-throughput bioluminescence-based mutant screening strategy for identification of bacterial virulence genes. Appl Environ Microb. 75(7): 2166–2175.

Kim DH and Rossi JJ. 2007. Strategies for silencing human disease using RNA interference. Nature Rev Genet. 8(3): 173–184.

Kreuze JF, Savenkov EI, Cuellar W, Li XD, and Valkonen JPT. 2005. Viral class 1 RNase III involved in suppression of RNA silencing. J Virology. 79(11): 7227–7238.

Kurath G. 2008. Biotechnology and DNA vaccines for aquatic animals. OIE Revue Scientifique et Technique. 27(1): 175–196.

Kurita J, Nakajima K, Hirono I, and Aoki T. 2002. Complete genome sequencing of the red seabream iridovirus. Fish Sci. 68(Suppl II): 1113–1115.

Kvitt H, Heinisch G, and Diamant A. 2008. Detection and phylogeny of Lymphocystivirus in sea bream *Sparus aurata* based on the DNA polymerase gene and major capsid protein sequences. Aquaculture. 275(1–4): 58–63.

Kwak KT, Gardner IA, Farver TB, and Hedrick RP. 2005. Rapid detection of sturgeon iridovirus (WSIV) using a polymerase chain reaction (PCR) assay. Aquaculture. 254(1–4): 92–101.

Lu L, Zhou SY, Chen C, Weng SP, Chan SM, and He JG. 2005. Complete genome sequence analysis of an iridovirus isolated from the orange-spotted grouper, *Epinephelus coioides*. Virology. 339(1): 81–100.

Lua DT, Yasuike M, Hirono I, and Aoki T. 2005. Transcription program of red seabream iridovirus as revealed by DNA microarrays. J Virology. 79(24): 15151–15164.

Lua DT, Yasuike M, Hirono I, Kondo H, and Aoki T. 2007. Transcription profile of red seabream iridovirus in a fish model as revealed by DNA microarrays. Virus Genes. 35: 449–461.

Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K, Iijima Y, Najima M, Nakano M, Yamashita A, Kubota Y, Kimura S, Yasunaga T, Honda T, Shinagawa H, Hattori M, and Iida T. 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. Lancet. 361(9359): 743–749.

Mao XL, Zhou S, Xu D, Gong J, Cui HC, and Qin QW. 2008. Rapid and sensitive detection of Singapore grouper iridovirus by loop-mediated isothermal amplification. J Appl Microbiol. 105(2): 389–397.

Marsden MJ, Devoy A, Vaughan LM, Foster TJ, and Secombes CJ. 1996. Use of a genetically attenuated strain of *Aeromonas salmonicida* to vaccinate salmonid fish. Aquacult Int. 4(1): 55–66.

Marsden MJ, Vaughan LM, Fitzpatrick RM, Foster TJ, and Secombes CJ. 1998. Potency testing of a live, genetically attenuated vaccine for salmonids. Vaccine. 16(11–12): 1087–1094.

Martin SAM, Blaney SC, Houlihan DF, and Secombes CJ. 2006. Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*). Mol Immunol. 43(11): 1900–1911.

Martin SAM, Collet B, MacKenzie S, Evensen O, and Secombes CJ. 2008. Genomic tools for examining immune gene function in salmonid fish. Res Fish Sci. 16(Suppl 1): 110–116.

Martin SAM, Mohanty BP, Cash P, Houlihan DF, and Secombes CJ. 2007. Proteome analysis of the Atlantic salmon (*Salmo salar*) cell line SHK-1 following recombinant IFN-γ stimulation. Proteomics. 7(13): 2275–2286.

Martyniuk CJ and Denslow ND. 2009. Towards functional genomics in fish using quantitative proteomics. Gen Comp Endocrinol. 164(2–3): 135–141.

Mehta PK, Pandey AK, Subbian S, El-Etr SH, Cirillo SL, Samrakandi MM, and Cirillo JD. 2006. Identification of *Mycobacterium marinum* macrophage infection mutants. Microb Pathogenesis. 40(4): 139–151.

Menéndez A, Fernández P, Reimundo P, and Guijarro JA. 2007. Genes required for *Lactococcus garvieae* survival in a fish host. Microbiol. 153(10): 3268–3294.

Midtlyng PJ and Lillehaug A. 1998. Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants. Dis Aquat Organ. 32(2): 91–97.

Millán A, Gómez-Tato A, Fernández C, Pardo BG, Álvarez-Dios JA, Calaza M, Bouza C, Vázquez M, Cabaleiro S, and Martínez P. 2009. Design and performance of a turbot (*Scophthalmus maximus*) oligomicroarray based on ESTs from immune tissues. Mar Biotechnol. 12(4): 452–465.

Miyata M, Inglis V, and Aoki T. 1996. Rapid identification of *Aeromonas salmonicida* subspecies *salmonicida* by the polymerase chain reaction. Aquaculture. 141(1–2): 13–24.

Mori Y and Notomi T. 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J Infect Chemother. 15(2): 62–69.

Mutoloki S, Alexandersen S, and Evensen Ø. 2004. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish Shellfish Immunol. 16(5): 633–644.

Nash JHE, Findlay WA, Luebbert CC, Mykytczuk OL, Foote SJ, Taboada EN, Carrillo CD, Boyd JM, Colquhoun DJ, Reith ME, and Brown LL. 2006. Comparative genomics profiling of clinical isolates of *Aeromonas salmonicida* using DNA microarrays. BMC Genomics. 7: 43.

Nilsson WB, Gudkovs N, and Strom MS. 2006. Atypical strains of *Aeromonas salmonicida* contain multiple copies of insertion element ISAsa4 useful as a genetic marker and a target for PCR assay. Dis Aquat Organ. 70(3): 209–217.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, and Hase T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acid Res. 28(12): E63.

Orešič M. 2009. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. Nutr Metab Cardiovas. 19(11): 816–824.

Pardo BG, Fernández C, Millán A, Bouza C, Vázquez-López A, Vera M, Alvarez-Dios JA, Calaz, M, Gómez-Tato A, Vázquez M, Cabaleiro S, Magariños B, Lemos ML, Leiro JM, and Martínez P. 2008. Expressed sequence tags (ESTs) from immune tissues of turbot (*Scophthalmus maximus*) challenged with pathogens. BMC Vet Res. 4: 37.

Pierce A, Unwin RD, Evans CA, Griffiths S, Carney L, Zhang L, Jaworska E, Lee CF, Blinco D, Okoniewski MJ, Miller CJ, Bitton DA, Spooncer E, and Whetton AD. 2008. Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. Mol Cell Proteom. 7(5): 853–863.

Poetsch A and Wolters D. 2008. Bacterial membrane proteomics. Proteomics. 8(19): 4100–4122.

Reith M, Singh RK, Curtis B, Boyd JM, Bouevitch A, Kimball J, Munholland J, Murphy C, Sarty D, Williams J, Nash JHE, Johnson SC, and Brown LL. 2008. The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. BMC Genomics. 9: 427.

Sample R, Bryan L, Long S, Majji S, Hoskins G. Sinning A, Olivier J, and Chinchar VG. 2007. Inhibition of iridovirus protein synthesis and virus replication by antisense morpholino oligonucleotides targeted to the major capsid protein, the 18 kDa immediate-early protein, and a viral homolog of RNA polymerase II. Virology 358(2): 311–320.

Samuelsson LM, Förlin L, Karlsson G, Adolfsson-Erici M, and Larsson DGJ. 2006. Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. Aquat Toxicol. 78(4): 341–349.

Samuelsson LM and Larsson DGJ. 2008. Contributions from metabolomics to fish research. Mol BioSyst. 4(10): 974–979.

Savan R, Kono T, Itami T, and Sakai M. 2005. Loop-mediated isothermal amplification: an emerging technology for detection of fish and shellfish pathogens. J Fish Dis. 28(10): 573–581.

Secombes C. 2008. Will advances in fish immunology change vaccination strategies? Fish Shellfish Immunol. 25(4): 409–416.

Seshadri R, Joseph SW, Chopra AK, Sha J, Shaw J, Graf J, Haft D, Wu M, Ren Q, Rosovitz MJ, Madupu

R, Tallon L, Kim M, Jin S, Vuong H, Stine OC, Ali A, Horneman AJ, and Heidelberg JF. 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966T: jack of all trades. J Bacteriol. 188(23): 8272–8282.

Shinmoto H, Taniguchi K, Ikawa T, Kawai K, and Oshima S-I. 2009. Phenotypic diversity of infectious red sea bream iriodvirus isolates from cultured fish in Japan. Appl Environ Microb. 75(11): 3535–3541.

Solanky KS, Burton IW, MacKinnon SL, Walter JA, and Dacanay A. 2005. Metabolic changes in Atlantic salmon exposed to *Aeromonas salmonicida* detected by H-nuclear magnetic resonance spectroscopy of plasma. Dis Aquat Organ. 65(2): 107–114.

Song WJ, Lin Q, Joshi SB, Lim TK, and Hew CL. 2006. Proteomic studies of the Singapore grouper Iridovirus. Mol Cell Proteom. 5(2): 256–264.

Song WJ, Qin QW, Qiu J, Huang CH, Wang F, and Hew CL. 2004. Functional genomics analysis of Singapore grouper iridovirus: complete sequence determination and proteomic analysis. J Virol. 78(22): 12576–12590.

Stentiford GD, Viant MR, Ward DG, Johnson PJ, Martin A, Wenbin W, Cooper HJ, Cooper HJ, Lyons BP, and Feist SW. 2005. Liver tumors in wild flatfish: A histopathological, proteomic, and metabolomic study. Omics. 9(3): 281–299.

Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PD, Abdellah Z, Arrowsmith C, Chillingworth T, Churcher C, Clarke K, Cronin A, Davis P, Goodhead I, Holroyd N, Jagels K, Lord A, Moule S, Mungall K, Norbertczak H, Quail MA, Rabbinowitsch E, Walker D, White B, Whitehead S, Small PL, Brosch R, Ramakrishnan L, Fischbach MA, Parkhill J, and Cole ST. 2008. Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. Genome Res. 8(5): 729–741.

Su Y-C, Wu J-L, and Hong J-R. 2009. Betanodavirus non-structural protein B2: a novel necrotic death factor that induces mitochondria-mediated cell death in fish cells. Virology. 385(1): 143–154.

Swift S, Karlyshev AV, Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S, and Stewart GSAB. 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the Luxri homologs AhyRi and AsaRi and their cognate N-acylhomoserine lactone signal molecules. J Bacteriol. 179(17): 5271–5281.

Swift S, Lynch MJ, Fish L, Kirke DF, Tomás JM, Stewart GSAB, and Williams P. 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun. 67(10): 5192–5199.

Teng Y, Hou Z, Gong J, Hong L, Xie X, Zhang L, Chen X, and Qin QW. 2008. Whole-genome transcriptional profiles of a novel marine fish iridovirus, Singapore grouper iridovirus (SGIV) in virus-infected grouper spleen cell cultures and in orange-spotted grouper, *Epinephulus coioides*. Virology. 377(1): 39–48.

Tidona CA and Darai G. 1997. The complete DNA sequence of lymphocystis disease virus. Virology. 230(2): 207–216.

Treasurer J and Cox C. 2008. Intraperitoneal and dorsal median sinus vaccination effects on growth, immune response, and reproductive potential in farmed Atlantic salmon *Salmo salar*. Aquaculture. 275(1–4): 51–57.

Tsai CT, Ting JW, Wu MH, Wu MF, Guo IC, and Chang CY. 2005. Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses. J Virol. 79(4): 2010–2023.

Tsoi SCM, Cale JM, Bird IM, Ewart V, Brown LL, and Douglas S. 2003. Use of human cDNA microarrays for identification of differentially expressed genes in Atlantic salmon liver during *Aeromonas salmonicida* infection. Mar Biotechnol. 5(6): 545–554.

Tsoi SCM, Ewart KV, Penny S, Melville K, Liebscher RS, Brown LL, and Douglas SE. 2004. Identification of immune-relevant genes from Atlantic salmon using suppression subtractive hybridization. Mar Biotechnol. 6(3): 199–214.

Viant MR, Bundy JG, Pincetich CA, de Ropp JS, and Tjeerdema RS. 2005. NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis. Metabolomics. 1(2): 149–158.

Viant MR, Rosenblum ES, and Tjeerdema RS. 2003. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. Environ Sci Technol. 37(21): 4982–4989.

von Schalburg KR, Cooper GA, Leong J, Robb A, Lieph R, Rise ML, Davidson WS, and Koop BF. 2008. Expansion of the genomics research on Atlantic salmon *Salmo salar* L. project (GRASP) microarray tools. J Fish Biol. 72(9): 2051–2070.

Wang F, Bi X, Chen LM, and Hew CL. 2008a. ORF018R, a highly abundant virion protein from Singapore grouper iridovirus, is involved in serine/

threonine phosphorylation and virion assembly. J Gen Virol. 89(5): 1169–1178.

Wang YQ, Lu L, Weng SP, Huang JN, Chan S-M, and He JE. 2007. Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus-like (ISKNV-Like) virus. Arch Virol. 152(4): 763–773.

Wang Z-L, Xu X-P, He B-L, Weng S-P, Xia J, Wang L, Lin T, Liu X, Wang Q, Yu X-Q, and He J-G. 2008b. Infectious spleen and kidney necrosis virus ORF48R functions as a new viral vascular endothelial growth factor. J Virol. 82(9): 4371–4383.

Wiens GD, Rockey DD, Wu Z, Chang J, Levy R, Crane S, Chen DS, Capri GR, Burnett JR, Sudheesh PS, Schipma MJ, Burd H, Bhattacharyya A, Rhodes LD, Kaul R, and Strom MS. 2008. Genome sequence of the fish pathogen *Renibacterium salmoninarum* suggests reductive evolution away from an environmental *Arthrobacter* ancestor. J Bacteriol. 190(21): 6970–6982.

Wiklund T and Dalsgaard I. 1998. Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. Dis Aquat Organ. 32(1): 49–69.

Winterberg KM and Reznikoff WS. 2007. Screening transposon mutant libraries using full-genome oligonucleotide microarrays. Method Enzymol. 421: 110–125.

Zenke K and Kim KH. 2008. Functional characterization of the RNase III gene of rock bream iridovirus. Arch Virol. 153(9): 1651–1656.

Zhang Q, Shi C, Huang J, Jai K, Chen X, and Lui H. 2009. Rapid diagnosis of turbot reddish body iridovirus in turbot using loop-mediated isothermal amplification method. J Virol Methods. 158(1–2): 18–23.

Zhang QY, Xiao F, Xie J, Li ZQ, and Gui JF. 2004. Complete genome sequence of lymphocystis disease virus isolated from China. J Virol. 78(13): 6982–6994.

Chapter 7

Antimicrobial Peptides and Their Potential as Therapeutants in Aquaculture

Susan E. Douglas

OVERVIEW

Teleost fish rely heavily on protective molecules of the innate immune system, of which antimicrobial peptides (AMPs) comprise one component. These small, usually cationic, amphipathic peptides are able to kill microbes very specifically. They are produced mainly by circulating immune cells and epithelial cells of the gut and skin and are often induced in response to infection. These peptides present a new avenue of disease prevention and/or treatment through their administration as therapeutants or via transgenic technology. Current treatments for combating disease in aquacultured organisms, recent advances in applications of AMPs, and future prospects in this field are described.

PHYSICAL PROPERTIES OF ANTIMICROBIAL PEPTIDES

AMPs (also known as host defense peptides) are small (usually less than 40 amino acids), gene-encoded peptides that are crucial components of the nonspecific innate immune system. They play a dual role in that they can effect killing of target cells as well as modulate innate and adaptive immune responses.

The mature active AMPs are processed from preproproteins, are generally rich in positively charged amino acids such as lysine and arginine, and contain up to 50% hydrophobic amino acids. Some AMPs contain modified amino acids such as dihydroxyarginine, dihydroxylysine, 6-bromotryptophan, and 3,4-dihydroxyphenylalanine (see Tincu and Taylor 2004). The carboxy terminus of AMPs, especially α–helical AMPs, is often amidated, which enhances helicity. Upon interaction of the positively charged amino acids with negatively charged components of biological membranes, AMPs assume an amphipathic conformation, in which the charged residues are well separated in space from the hydrophobic residues. This allows disruption or penetration of the membrane and a variety of mechanisms have been proposed (Reddy et al. 2004; Jenssen et al. 2006).

This review focuses on small cationic AMPs isolated from marine organisms, since these have coevolved with their hosts to combat pathogens while sparing beneficial commensals. Furthermore, small peptides and derivatives are more easily and affordably produced as therapeutic agents. Larger AMPs (>7 kDa) such as crustins that have been described

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

from crustaceans will not be discussed (for review, see Smith et al. 2008). Similarly, AMPs that comprised fragments of larger positively charged biological molecules such as histones (Park et al. 1998; Robinette et al. 1998; Richards et al. 2001; Birkemo et al. 2003; Li et al. 2007), hemoglobin (Ullal et al. 2008), and ribosomal (Fernandes and Smith 2002) and other proteins (Fernandes et al. 2003) will not be discussed in this review.

AMPs can be broadly classified into three categories: α-helical, open, and closed loops containing disulphide-bonded β-sheets, and extended structures with a predominance of a single amino acid (usually tryptophan, proline, histidine).

The α-helical AMPs include insect-derived cecropins, amphibian-derived magainins, and fish-derived pleurocidins (Cole et al. 1997; Douglas et al. 2001), chrysophins (Iijima et al. 2003), piscidins (Silphaduang and Noga 2001; Silphaduang et al. 2006; Noga et al. 2009), moronecidins (Lauth et al. 2002), misgurnin (Park et al. 1997), pardaxin (Lazarovici et al. 1986; Shai et al. 1988), and cathelicidins (Uzzell et al. 2003; Chang et al. 2006). Within the invertebrate kingdom, α-helical AMPs are less common although styelins (Lee et al. 1997a), dicynthaurin (Lee et al. 2001a), halocidin (Jang et al. 2002), and histidine-rich clavanins (Lee et al. 1997b) and clavaspirin (Lee et al. 2001b) from tunicate hemocytes are all α-helical.

The β-sheet class of AMPs includes the cysteine-rich highly disulphide-bonded peptides such as defensins (Saito et al. 1995; Charlet et al. 1996; Mitta et al. 1999b), mytilins (Charlet et al. 1996; Mitta et al. 2000a), myticins (Mitta et al. 1999a), mytimycins (Mitta et al. 2000c), tachyplesins (Nakamura et al. 1988), polyphemusins (Miyata et al. 1989), and hepcidins (Shike et al. 2002; Douglas et al. 2003a; Ren et al. 2006; Yang et al. 2007; Cho et al. 2009). Recently, a beta defensin-like AMP was discovered in rainbow trout using in silico prediction methods (Falco et al. 2008b).

A third class of AMPs includes extended structures with a predominance of a single amino acid. Proline-rich AMPs include astacidin 2 from hemocytes of crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al. 2007) and a 6.5 kDa AMP with close similarity to bovine bactenicin 7 from shore crab, *Carcinus maenas* (Schnapp et al. 1996). Partial sequence information for callinectin, a 3.7 kDa AMP isolated from blue crab, *Callinectes sapidus*, indicates that it contains substantial amounts of arginine and proline, but they are not arranged in the motifs typical of known proline-rich AMPs (Khoo et al. 1999). Shrimp penaeidins (Bachere et al. 2004) and arasin, a 37 amino acid AMP isolated from the small spider crab, *H. araneus* (Stensvag et al. 2008), possess a chimeric structure characterized by a linear N-terminal domain rich in proline and arginine followed by a C-terminal domain containing six cysteines.

DISTRIBUTION OF ANTIMICROBIAL PEPTIDES

AMPs are widely distributed in nature, occurring in a vast number of organisms including plants, animals, and bacteria. At the time of writing, 1345 AMPs have been deposited in the Antimicrobial Peptide Database (APD2) (http://aps.unmc.edu/AP/main.php), including those from 48 marine fish species and 39 invertebrates.

Because fish and invertebrates are constantly bathed in microbe-containing water, they rely on their innate immune defenses, including AMPs present in the protective mucous secretions. Half of the global biodiversity is found in the oceans, making marine organisms a valuable resource for the discovery of AMPs and other novel bioactive compounds. Exploration of deep sea hydrothermal vents have proved fruitful in the discovery of a new mytilin from the bivalve, *Bathymodiolus azoricus* (Bettencourt et al. 2007). In addition, novel AMPs have been isolated from bacteria living on fish (Sirtori et al. 2006).

A huge diversity of AMPs from marine invertebrates has been described. Penaeidins have been isolated from many species of shrimp (for review, see Cuthbertson et al. 2008). A comprehensive database (PenBase 2010) has been developed for the penaedins (http://www.penbase.immunaqua.com) (Gueguen et al. 2006a). In addition, defensins, myticin, mytilin, and mytimycin have been isolated from molluscs, tachyplesins, and polyphemusins from chelicerates, and clavanins and styelins from tunicates (for review, see Tincu and Taylor 2004). A recent report identified a cysteine-rich AMP from green sea urchin *Strongylocentrotus droebachiensis* (Li et al. 2008).

Recently, genomics approaches such as EST surveys and suppression subtractive hybridization (SSH) have uncovered additional AMPs, some with unique properties such as salt tolerance. EST surveys of such organisms as black tiger shrimp *Penaeus monodon* (Supungul et al. 2002, 2004; Tassanakajon et al. 2006), Chinese shrimp *Fenneropenaeus chinensis* (Shen et al. 2004; Dong and Xiang 2007), Pacific blue shrimp *Litopenaeus stylirostris* (de Lorgeril et al. 2005), Pacific white shrimp *Litopenaeus vannemei* (Gross et al. 2001), Pacific oyster *Crassostrea gigas* (Gueguen et al. 2003), clam *Ruditapes decussatus* (Gestal et al. 2007), and four other bivalves (Tanguy et al. 2008) have revealed many new AMP sequences. A novel reverse genetics approach was used to identify AMP ESTs in hemocyte libraries from the sea squirt *Ciona intestinalis* (Fedders et al. 2008). Searchable databases for *P. monodon* (http://pmonodon.biotec.or.th/), *L. stylirostris* (http://www.ifremer.fr/StyliBase/), and *C. gigas* (http://www.ifremer.fr/GigasBase/) have been created to facilitate organization of these ESTs.

An important finding from these studies is that many AMPs exist as gene families, and many sequence variants exist (Douglas et al. 2003b; Patrzykat et al. 2003; Bachere et al. 2004; Supungul et al. 2004; Pallavicini et al. 2008; Cho et al. 2009). These variants or isoforms are often differentially expressed according to tissue, developmental stage, physiological condition, or disease status.

EXPRESSION OF ANTIMICROBIAL PEPTIDES

The expression of AMPs is often tissue-specific, with the highest expression in fish existing in liver (Douglas et al. 2003a), epithelial cells lining the gut (Douglas et al. 2001), and skin (Cole et al. 1997), as well as in circulating granulocyte immune cells (Iijima et al. 2003; Murray et al. 2003, 2007; Mulero et al. 2008a). Invertebrate AMPs are mainly expressed in hemocytes (Bachere 2003), but enterocytes have also been shown to contain mytilin B (Mitta et al. 2000b), and defensins have been identified in the mantle cells of oysters (Gueguen et al. 2006b).

Expression of AMPs in invertebrates is especially important as they lack antibody-mediated protective immunity (Bachere 2003). Most invertebrate AMPs are constitutively expressed and are important in maintaining the normal bacterial flora in healthy organisms. There is seldom transcriptional regulation of penaeidin expression in shrimp exposed to microbial challenge (Destoumieux et al. 2000). Instead, hemocytes migrate to sites of infection where they exert their effects against invading pathogens by releasing AMPs from the granules (Bachere et al. 2004; Munoz et al. 2004). Subsequent proliferation of hemocytes occurs with concomitant increase in the amount of AMPs released by degranulation. The presence of AMPs in the plasma of infected mussels indicates that they may play a secondary systemic role in combating infection (Mitta et al. 2000a).

In contrast to invertebrates, the expression of many fish AMPs is induced under conditions of stress or disease (Douglas et al. 2003a; Chiou et al. 2007a; Cho et al. 2009). Promoters known to be involved in expression of other mediators of innate immunity have been identified upstream of AMP genes in a number of organisms (Douglas et al. 2003b; Cho et al. 2009).

The expression of AMPs is often developmentally controlled, with some variants being expressed early in development and others at later stages (Douglas et al. 2001). Since larval fish are unable to mount an adaptive immune response, the expression of AMPs and other innate immune effectors early in development is a crucial protective mechanism (Mulero et al. 2008b). The expression of AMPs in developing shrimp is particularly important during the molting stages as the organisms are especially susceptible to infection at these times (Munoz et al. 2003; Chiou et al. 2007b).

ACTIVITIES OF ANTIMICROBIAL PEPTIDES

Antibacterial Activity

Most AMPs exert a direct cytotoxic effect on bacterial cells in the extracellular space. However, some AMPs also participate in intracellular killing within the bacteria-containing phagosome of immune cells (Mitta et al. 2000a, 2000b; Mulero et al. 2008a) or oposonize the bacterial cell for subsequent phagocytosis by specialized hyaline cells (Munoz et al. 2002).

The majority of fish AMPs exert rapid, broad-spectrum killing of both Gram-positive (G+) and Gram-negative (G−) bacteria. This is usually accomplished through disruption of the bacterial cell membrane causing lysis or by translocation into the cell where intracellular targets are inhibited. The antibacterial specificities of invertebrate AMPs are more variable and have been reviewed in Tincu and Taylor (2004). To summarize, penaeidins mainly kill or inhibit G+ bacteria, whereas halocidin, astacidin, clavanin, clavaspirin, styelin, tachyplesin, and polyphemusin are more broad spectrum and target both G+ and G− bacteria.

Antiviral Activity

A number of AMPs exhibit antiviral activity (for review, see Jenssen et al. 2006). This activity can be due to the ability of positively charged AMPs to bind to negatively charged proteoglycans or specific cellular receptors such as CXCR4 (Fujii and Tamamura 2001) needed for viral entry. Some AMPs can bind to viral envelope glycoproteins or alter host cell membrane composition, thereby inhibiting viral entry. Yet, others can interact with host intracellular targets, activating host antiviral mechanisms or blocking viral gene or protein expression. Finally, AMPs can inhibit viral spread across tight junctions or through syncytium formation.

Little information is available on antiviral effects of marine fish and invertebrate AMPs (Table 7.1). Tachyplesin exerts direct effects on the envelope of vesicular stomatitis virus, influenza A virus (Murakami et al. 1991), and HIV (Morimoto et al. 1991). A synthetic polyphemusin-based AMP has been shown to inhibit syncytium formation by binding to the HIV envelope gp120 protein and/or T-cell surface CD4 (Tamamura et al. 1996). The recent identification of a fish CD4 homolog indicates that a similar mechanism could operate in fish (Laing et al. 2006). Mussel defensins and fragments of defensin are active against HIV-1 (Roch et al. 2004), and synthetic mytilin added to white spot syndrome virus (WSSV) prior to exposure of shrimp is able to prevent in vivo infection by inhibiting viral DNA replication (Dupuy et al. 2004).

The insect-derived cecropin and a synthetic analog are able to kill a number of fish viral pathogens, including infectious hematopoietic necrosis virus, viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus, and infectious pancreatic necrosis virus (IPNV) in the Chinook salmon embryonic cell line CHSE-214 and carp cell line EPC (Chiou et al. 2002). For the enveloped viruses, inhibition of viral replication results from direct disruption of the viral membrane, probably via interactions with viral proteins in the envelope. However, for the nonenveloped IPNV, another mechanism such as disintegration of viral capsids or binding to viral receptors in the host cells may be involved. Human alpha defensin 1 was recently shown to inactivate VHSV by interfering with VHSV-G protein-dependent fusion and by inhibiting viral replication through the induction of Mx genes (Falco et al. 2007). A recombinant rainbow trout beta defensin expressed in a fish cell line was able to protect against VHSV, again through induction of Mx genes (Falco et al. 2008b). Four different piscidins from hybrid striped bass rapidly inactivate channel catfish virus and frog virus 3 and are effective over a wide temperature range (Chinchar et al. 2004).

Antifungal Activity

AMPs can kill fungi by lysing the cells or interfering with cell wall synthesis, chitin, or glucan biosynthesis (de Lucca and Walsh 1999). The carboxy terminus of penaeidins has similarities to chitin-binding proteins (see Bachere et al. 2004), which may explain their ability to inhibit growth of three filamentous fungi, *Fusarium oxysporum*, *Botrytis cinerea,* and *Penicillium crustosum* (Cuthbertson et al. 2004), as well as antibiotic-resistant *Cryptococcus* and *Candida* strains (Cuthbertson et al. 2006). The amino terminus of tachycitin contains a chitin-binding region that confers antifungal activity (Kawabata et al. 1996). Tachyplesin and polyphemusins are also able to inhibit fungal growth (Miyata et al. 1989). Of the molluscan AMPs, mytimycin is strictly antifungal, whereas only certain forms of mytilin are able to inhibit *F. oxysporum* (Mitta et al. 2000c). A portion of the mussel MGD1 defensin is active against *F. oxysporum* (Romestand et al. 2003).

Some pleurocidin and piscidin isoforms are able to inhibit the growth of *Candida albicans* (Patrzykat et al. 2003; Sung et al. 2008) and act by lysing the fungal cell membrane (Jung et al. 2007). A synthetic peptide derived from halocidin is active against

Table 7.1. Distribution and Spectrum of Activity of Antimicrobial Peptides from Marine Fish and Invertebrates.

Peptide	Source	Activity	Reference
Molluscs			
Defensin	*Mytilis edulis*	G+, (G−), V, P	Roch et al. 2004
MGD-1,2	*Mytilis galloprovencialis*	G+, F	Romestand et al. 2003
Mytilin	*M. edulis*	G+, G−, F, V	Roch et al. 2008
Myticin	*M. edulis*	G+, (G−), F	Mitta et al. 1999a
Mytimycin	*M. edulis*	F	Mitta et al. 2000c
Crustaceans			
Penaeidins	Many	G+, F	Bachere et al. 2004
Carcinin	*Carcinus maenas*	G+, G−	Schnapp et al. 1996
Callinectin	*Callinectes sapidus*	G−	Khoo et al. 1999
Chelicerates			
Tachyplesin	*Tachyplesus tridentatus*	G+, G−, F, H	Miyata et al. 1989
		V	Murakami et al. 1991
		P	Morvan et al. 1997
Big defensin	*T. tridentatus*	G+, G−	Saito et al. 1995
Tachycitin	*T. tridentatus*	G+, G−, F	Kawabata et al. 1996
Polyphemusin	*Limulus polyphemus*	G+, G−, F	Miyata et al. 1989
		V	Tamamura et al. 1996
Tunicates			
Clavanin	*Styela clava*	G+, G−, F	Lee et al. 1997b
Clavispirin	*S. clava*	G+, G−, H	Lee et al. 2001b
Styelin	*S. clava*	G+, G−, H	Lee et al. 1997a
Halocidin	*Halocynthia aurantium*	G+, G−, F	Jang et al. 2002, 2006
Fish			
Misgurnin	*Misgurnus anguillicaudatus*	G+, G−, F	Park et al. 1997
Pleurocidins	Pleuronectid flatfish	G+, G−, F	Patrzykat et al. 2003
Moronecidin	*Morone chrysops*	G+, G−, F	Lauth et al. 2002
Piscidins	Many perciformes	G+, G	Silphadung and Noga 2001
		P	Colorni et al. 2008
		V	Chinchar et al. 2004
		F	Sung et al. 2008
Chrysophin	*Chrysophrys major*	G+, G−	Iijima et al. 2003
Pardaxin	*Pardachirus pavoninus*	G+, G−, H	Shai et al. 1988
Pardaxin	*P. marmoratus*	G+, G−, H	Lazarovicci et al. 1996
Cathelicidin	Hagfish	G+, G−	Uzzel et al. 2003
Cathelicidin	Salmonids	G+,G−	Chang et al. 2006
Hepcidin	Many	G+, G−, F	Bulet et al. 2004

G+, Gram-positive bacteria; G−, Gram-negative bacteria; F, fungi; V, viruses; P, parasites; H, hemolytic. Terms in brackets indicate limited activities.

several strains of *Candida* and *Aspergillus* (Jang et al. 2006). This peptide bound to β-1,3-glucan in the fungal cell wall and induced the formation of ion channels in the membrane.

Antiparasitic Activity

Few studies have investigated the antiparasitic activities of AMPs, and those that have mainly focused on histone-like proteins or AMPs from nonmarine organisms (see Colorni et al. 2008). Early studies showed that tachyplesin I was able to kill several bivalve parasites, including the oyster pathogens *Bonamia ostreae* and *Perkinsus marinus* while sparing the host cells (Morvan et al. 1997). Recently, piscidin 2 has been shown to rapidly kill the ciliates *Ichthyophthirius multifiliis, Cryptocaryon irritans,* and *Trichodina* sp., and the dinoflagellate *Amyloodinium ocellatum*, all aquaculturally relevant pathogens (Colorni et al. 2008). Furthermore, killing occurred at concentrations that are normally found in gill tissue of fish (approximately 40 ug mL^{-1}). Mussel defensins and fragments were active against *Trypanosoma brucei* and *Leishmania major* (Roch et al. 2004). In this study, positive charge and small size (9 amino acids) were the key factors affecting antiprotozoan activity, and activity was mediated by generalized binding to the cell membrane and penetration.

Cytotoxic Activity

Some AMPs such as melittin from bees can be highly hemolytic or cytotoxic toward eukaryotic cells. However, few AMPs from marine fish and invertebrates exhibit this activity (Table 7.1). Interestingly, although tachyplesin is hemolytic, cyclized versions have reduced hemolytic activity (Bulet et al. 2004). Clavispirin is almost as hemolytic as melittin (Lee et al. 2001b), and piscidins show reduced hemolytic activity compared to melittin (Silphaduang and Noga 2001). Pleurocidin showed minimal hemolytic activity and was nontoxic to human intestinal epithelial cells at concentrations used to kill foodborne bacterial and fungal pathogens (Burrowes et al. 2004). This property makes it an excellent natural alternative to antibiotics used in aquaculture and current chemical preservatives used to ensure food safety.

Immunomodulatory Activity

In addition to their ability to directly kill pathogens, AMPs also act as signaling molecules, mobilizing the host defense system, attracting immune cells to sites of infection, and even stimulating the mounting of an adaptive immune response (Brown and Hancock 2006). The possibility of enhancing nonspecific defenses using AMPs in aquacultured fish and shellfish presents an interesting application for therapeutic treatment. A recent study in trout showed that intramuscular injection of synthetic HNP1 caused chemotaxis of leukocytes and induced the expression of genes encoding both proinflammatory cytokines and CC chemokines as well as of genes involved in type I interferon production in muscle, head kidney, and blood (Falco et al. 2008a).

THERAPEUTIC POTENTIAL OF ANTIMICROBIAL PEPTIDES

Infectious diseases caused by bacteria, fungi, viruses, and parasites (both protozoan and multicellular) are major threats the aquaculture industry is facing. Because aquacultured organisms often live in suboptimal nutritional and environmental situations and are exposed to pollution, their susceptibility to disease is increased. The use and overuse of antibiotics has led to problems with antibiotic resistance, not only for the fish but also for the human consumers. Therefore, alternative chemotherapeutants such as AMPs must be developed that do not present harm to the consumer or cause the emergence of resistant microbial mutants.

Current Approaches to Control of Disease in Aquaculture

Stimulation of the innate immune system using probiotics and immunostimulants such as alginates, β-glucans, or nucleic acids is currently used to control disease outbreaks in aquaculture settings (see Dalmo and Bogwald 2008). Live probiotics are thought to exert their beneficial effects by competing with harmful bacteria, producing inhibitory molecules or stimulating the immune system by increasing granulocyte numbers and enhancing phagocytosis (Bachere 2003). A recent study with rohu fish showed that orally administered *Bacillus*

subtilis resulted in significantly higher respiratory burst activity in neutrophils, higher serum bactericidal activity, and higher granulocyte numbers in treated fish than controls (Kumar et al. 2008). Inactivated bacteria, which present less environmental risk in aquaculture settings, can also be effective probiotics (Salinas et al. 2008). The use of β-glucan immunostimulants is now widespread, with many aquaculture feed producers incorporating it into diets (see Dalmo and Bogwald 2008). The administration of β-glucan resulted in a significant increase in nitric oxide and respiratory burst in mussels and higher antibacterial activity in hemolymph of clams (Mar Costa et al. 2008).

Stimulation of the adaptive immune system using vaccines is used against bacterial pathogens that cause such diseases as pasteurellosis, vibriosis, and edwardsiellosis, and a number of strategies for successful immunization have been implemented (see Secombes 2008). However, even with access to vaccines, these are only effective with adults; a recent study showed that vaccination of gilthead seabream larvae against *Photobacterium damselae* subsp. *piscicida* either by immersion or orally resulted in increased susceptibility to infection rather than protection (Mulero et al. 2008b). Furthermore, it is not practical to develop vaccines against every potential pathogen, so more generic protection against a wide variety of pathogens would be valuable. Vaccines against viral pathogens are difficult to develop and only one subunit vaccine against IPNV has been approved (see Chiou et al. 2002). DNA vaccines have been used with varying degrees of success against a variety of bacterial and viral fish pathogens (for review, see Tonheim et al. 2008). They have been particularly useful in treating viral diseases and have the advantage of stimulating both innate and adaptive immunity.

Control of Disease Using Antimicrobial Peptides

AMPs are attractive candidates for alternative therapeutants as they kill target organisms quickly, exhibit a broad spectrum of activity, and are highly selective (Sarmasik and Chen 2003). Although bacteria have evolved various mechanisms to avoid exposure to or killing by AMPs (Yeaman and Yount 2003; Sallum and Chen 2008), the development of resistant mutants is rare (Kraus and Peschel 2006). This is probably due to the proposed "multi-hit" mechanism of action (Powers and Hancock 2003), whereby AMPs are able to bind to and inhibit a number of different anionic molecules, making it difficult to induce resistance. Several AMPs are under development for human applications (for review, see Zaiou 2007) and it seems likely that AMPs will soon be applied to disease control in aquaculture.

Given their multifunctional roles in immunity, AMPs could be used to both stimulate nonspecific immune responses against pathogens in a generalized fashion as well as to directly kill pathogens. However, in any regime in which AMPs are used to control disease, care must be taken not to disturb the normal gut flora. Probiotic supplementation may be necessary to restore the gut microbiota (Gomez and Balcazar 2008). An alternative approach is to use "smart" peptides that selectively target the pathogen and leave the commensals unaffected (Eckert et al. 2006). Although AMPs from any source could potentially be used as therapeutic agents in aquaculture, this review will focus on those that are effective against fish or shellfish pathogens or are of marine fish or invertebrate origin.

Direct Administration

AMPs can be administered by immersion, injection, or orally in feed. In all cases, care must be taken to minimize inactivation by proteases, binding proteins, and salt. Although insect-derived cecropins B and P1 were effective against five fish bacterial pathogens, they were inhibited in the presence of NaCl over 0.1 M (Kjuul et al. 1999). On the other hand, pleurocidin (Patrzykat et al. 2003) and styelins (Lee et al. 1997a) show less sensitivity to salt, the latter tolerating up to 0.4 M NaCl.

Early studies showed that several AMPs protected salmon from *Vibrio anguillarum* infection (Jia et al. 2000), and more recent studies have shown that penaeidins can protect shrimp from pathogenic vibrios (Munoz et al. 2004). A ten amino acid peptide based on mytilin that formed a stable β-hairpin structure was able to protect shrimp from WSSV infection (Roch et al. 2008), and injection of a truncated version of epinecidin protected grouper and tilapia from *Vibrio vulnificus* infection (Pan et al. 2007).

Immersion treatment of young trout with β-glucans increased nonspecific disease resistance

(Jeney and Anderson 1993) and immersion treatment with AMPs may similarly enhance the innate immune response. Various immunostimulants have been administered to fish in feed but appropriate dosing regimens must be optimized and long-lasting additives must be developed for this approach to be feasible in practice (for review, see Raa 1996). Currently, attempts are underway to enhance shrimp resistance to disease by including a yeast strain producing recombinant AMP (Lu, personal communication).

Vaccine Adjuvants

Traditional adjuvants in fish vaccines include alum and oil. However, molecules such as cytokines, which activate dendritic cells and cause lymphocyte differentiation, have been successfully used as vaccine adjuvants in mammalian systems and show promise for fish and shellfish (see Secombes 2008). For example, a mouse cathelicidin-like AMP promoted both humoral and cellular antigen-specific immune responses when coadministered with OVA antigen to mice (Kurosaka et al. 2005). It would be of interest to see if any of the fish-derived cathelicidins also have this ability. Pleurocidin induced the expression of IL-1β in trout macrophages (Chiou et al. 2006) and IL-1β, TNF-α1, and IL-8 in trout in vivo (Falco et al. 2008a). HNP1 modulated the expression of IL-1β in blood and several other proinflammatory cytokines and chemokines in muscle as well as Mx genes in head kidney (Falco et al. 2008a). This opens the door for the use of fish-derived AMPs that stimulate cytokine expression as potential adjuvants in aquaculture.

DNA Vaccines

Just as coadministration of cytokines with vaccines can enhance the immune response, the generation of chimeric DNA vaccines containing cytokines such as IL-8 have been successful (Kornbluth and Stone 2006). Using the same rationale, AMPs that induce cytokine expression could form the basis for similar chimeric DNA vaccines. An advantage of DNA vaccines is that constructs encoding multiple AMPs that act synergistically could be designed.

Genetic Selection for Enhancement of Disease Responses

ESTs overexpressed in strains able to survive experimentally induced infection could be predictive biomarkers for selection programs or surveys of disease incidence in aquaculture. In shrimp surviving challenge with *Vibrio penaeicida*, ESTs corresponding to penaeidins were overrepresented in SSH cDNA libraries (de Lorgeril et al. 2005). By selecting for animals overexpressing AMPs, disease-resistant strains may be obtained. Interestingly, higher levels of penaeidin mRNA expression and higher numbers of penaeidin-expressing hemocytes were seen in third generation shrimp selected for survival against *V. penaeicida* than in control nonselected animals (de Lorgeril et al. 2008). In addition, higher amounts of penaeidin were present in both control and selected shrimp surviving the *V. penaeicida* challenge than in those that died.

Transgenic Expression

The development of transgenic fish and shellfish expressing AMP genes promises to yield disease-resistant species for aquaculture. Experimental proof for this concept was obtained in channel catfish expressing the insect-derived AMP, cecropin (Dunham et al. 2002). Transgenic catfish showed improved resistance (40.7%) to *Edwardsiella ictalurii* compared to nontransgenic control animals (14.8%). Cecropin transgenes expressed in stably transfected CHSE-214 fish cells (Sarmasik and Chen 2003) and medaka (Sarmasik et al. 2002) were able to protect against several fish pathogens, including *Aeromonas hydrophila, Pseudomonas fluorescens,* and *V. anguillarum*. A beta defensin-like AMP transfected into a trout cell line was able to protect against VHSV (Falco et al. 2008b) and pleurocidin-encoding constructs injected into trout resulted in expression of active pleurocidin (Brocal et al. 2006; Falco et al. 2008a). Together with the finding that a pleurocidin sequence stably transfected into the carp EPC cell line was expressed and secreted for over two years (Brocal et al. 2006), this indicates that pleurocidin and perhaps other AMPs may be successfully produced in transgenic fish. It is even conceivable that transgenic fish could be used

as biofactories to express AMPs of therapeutic interest (Rocha et al. 2003).

FUTURE DEVELOPMENTS

While naturally occurring AMPs are active against various microbial pathogens of aquacultured species, there are a number of drawbacks that must be addressed. These include susceptibility to protease degradation, salt-dependent inactivation, potential host toxicity (including hemolysis), delivery, and cost of production (Hancock and Sahl 2006). Although several AMPs are in clinical trials for various applications in humans, none have yet been approved by the FDA for clinical use. Applications for therapy in aquacultured species, while they hold great promise, are still in their infancy.

Various modifications have been introduced to decrease degradation of AMPs. Replacement of *L*-amino acids by *D*-amino acids or nonstandard amino acids, addition of protecting groups, introduction of disulphide bonds, cyclization, truncation, and development of peptidomimetics (Freidinger 2003) are several approaches that have been explored to enhance stability (see Jenssen et al. 2006). Efficacy and selectivity can be augmented by modifying the hydrophobicity or net charge of AMPs, or by replacing specific amino acid residues to promote amphipathicity.

Synthetic truncated mussel defensins as well as fragments containing amino acid replacements were able to kill the protozoan parasites, *T. brucei* and *L. major*, and the HIV-1 virus more effectively than the native forms (Roch et al. 2004). Truncation of penaeidins to produce only the PRD (proline-rich domain) results in an AMP that is up to five times more effective than full-length penaeidin (Cuthbertson et al. 2006). A synthetic ten amino acid fragment of mytilin constrained by two disulphide bonds into a stable β-hairpin showed improved antiviral and antibacterial activity and has been suggested as a feed additive for marine invertebrates (Roch et al. 2008). Addition of a lysine residue to the amino terminus of halocidin improved antimicrobial activity while reducing hemolytic activity (Jang et al. 2003).

In order to prevent degradation of AMPs, particularly those administered orally, delivery methods using chitosan or alginate microparticles could be developed. This approach has been successfully used for delivery of DNA vaccines (Rajesh Kumar et al. 2008; Tian et al. 2008).

For AMPs to be used commercially in aquaculture, the cost of production must be significantly reduced. Recombinant expression of AMPs or modified derivatives has been successfully undertaken using different strategies for heterologous expression (for review, see Ingham and Moore 2007). Pleurocidin has been successfully produced in both *E. coli* (Bryksa et al. 2006) and a fish cell line (Brocal et al. 2006) and recombinant fish hepcidins have been expressed in *E. coli* (Greenshields et al. 2008; Srinivasulu et al. 2008). An oyster defensin has been expressed in *E. coli* (Gueguen et al. 2006b) and various penaeidins have been expressed in yeast systems (Destoumieux et al. 1999; Li et al. 2005).

In conclusion, the broad-spectrum activity of marine-derived AMPs combined with their limited hemolytic and cytotoxic activity make them excellent candidates as therapeutics in aquaculture. Coupled with their ability to kill many bacteria, yeasts and molds of importance to food safety, AMPs are also ideal candidates for food preservation in aquaculture. Proof-of-concept research has been conducted to produce recombinant or synthetic variants with enhanced properties for protecting fish and shellfish against disease. The future looks promising for the application of these peptides in the aquaculture industry.

ACKNOWLEDGMENTS

I would like to thank Dr. J. K. Lu for permission to cite his unpublished work, and Dr. Michael Reith for critical review of this manuscript. Also, I am indebted to all my colleagues who have contributed to our research on pleurocidins and hepcidins, especially Jeff Gallant, Harry Murray, and Aleks Patrzykat. This is NRC publication number NRCC 2009-42819.

REFERENCES

Bachere E. 2003. Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. Aquaculture. 227: 427–438.

Bachere E, Gueguen Y, Gonzalez M, de Lorgeril J, Garnier J, and Romestand B. 2004. Insights into the anti-microbial defense of marine invertebrates: the

penaeid shrimps and the oyster *Crassostrea gigas*. Immunol Rev. 198: 149–168.

Bettencourt R, Roch P, Stefanni S, Rosa D, Colaco A, and Santos RS. 2007. Deep sea immunity: unveiling immune constituents from the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar Environ Res. 64: 108–127.

Birkemo AG, Luders T, Andersen O, Nes IF, and Nissen-Meyer J. 2003. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). Biochim Biophys Acta. 1646: 207–215.

Brocal I, Falco A, Mas V, Rocha A, Perez L, Coll JM, and Estepa A. 2006. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Appl Microbiol Biotechnol. 72: 1217–1228.

Brown KL and Hancock RE. 2006. Cationic host defense (antimicrobial) peptides. Curr Opin Immunol. 18: 24–30.

Bryksa BC, MacDonald LD, Patrzykat A, Douglas SE, and Mattatall NR. 2006. A C-terminal glycine suppresses production of pleurocidin as a fusion peptide in *Escherichia coli*. Prot Expr Purif. 45: 88–98.

Bulet P, Stocklin R, and Menin L. 2004. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev. 198: 169–184.

Burrowes OJ, Hadjicharalambous C, Diamond G, and Lee T-C. 2004. Evaluation of antimicrobial spectrum and cytotoxic activity of pleurocidin for food applications. J Food Sci. 63: 66–71.

Chang CI, Zhang YA, Zou J, Nie P, and Secombes CJ. 2006. Two cathelicidin genes are present in both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Antimicrob Ag Chemother. 50: 185–195.

Charlet M, Chernysh S, Philippe H, Hetru C, Hoffmann JA, and Bulet P. 1996. Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J Biol Chem. 271: 21808–21813.

Chinchar VG, Bryan L, Silphadaung U, Noga E, Wade D, and Rollins-Smith L. 2004. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. Virology. 323: 268–275.

Chiou P, Khoo J, Bols NC, Douglas S, and Chen TT. 2006. Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. Dev Comp Immunol. 30: 797–806.

Chiou PP, Lin CM, Bols NC, and Chen TT. 2007a. Characterization of virus/double-stranded RNA-dependent induction of antimicrobial peptide hepcidin in trout macrophages. Dev Comp Immunol. 31: 1297–1309.

Chiou PP, Lin CM, Perez L, and Chen TT. 2002. Effect of cecropin B and a synthetic analogue on propagation of fish viruses *in vitro*. Mar Biotechnol. 4: 294–302.

Chiou TT, Lu JK, Wu JL, Chen TT, Ko CF, and Chen JC. 2007b. Expression and characterisation of tiger shrimp *Penaeus monodon* penaeidin (mo-penaeidin) in various tissues, during early embryonic development and moulting stages. Dev Comp Immunol. 31: 132–142.

Cho YS, Lee SY, Kim KH, Kim SK, Kim DS, and Nam YK. 2009. Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin isoforms resulting from different biological stimulations. Dev Comp Immunol. 33: 46–58.

Cole AM, Weis P, and Diamond G. 1997. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J Biol Chem. 272: 12008–12013.

Colorni A, Ullal A, Heinisch G, and Noga E-J. 2008. Activity of the antimicrobial polypeptide piscidin 2 against fish ectoparasites. J Fish Dis. 31: 423–432.

Cuthbertson BJ, Bullesbach EE, Fievet J, Bachere E, and Gross PS. 2004. A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. Biochem J. 381: 79–86.

Cuthbertson BJ, Bullesbach EE, and Gross PS. 2006. Discovery of synthetic penaeidin activity against antibiotic-resistant fungi. Chem Biol Drug Des. 68: 120–127.

Cuthbertson BJ, Deterding LJ, Williams JG, Tomer KB, Etienne K, Blackshear PJ, Bullesbach EE, and Gross PS. 2008. Diversity in penaeidin antimicrobial peptide form and function. Dev Comp Immunol. 32: 167–181.

Dalmo RA and Bogwald J. 2008. Beta-glucans as conductors of immune symphonies. Fish Shellfish Immunol. 25: 384–396.

de Lorgeril J, Gueguen Y, Goarant C, Goyard E, Mugnier C, Fievet J, Piquemal D, and Bachere E. 2008. A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection. Mol Immunol. 45: 3438–3445.

de Lorgeril J, Saulnier D, Janech MG, Gueguen Y, and Bachere E. 2005. Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an

infection with *Vibrio penaeicida*. Physiol Genomics. 21: 174–183.

De Lucca AJ and Walsh TJ. 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob Ag Chemother. 43: 1–11.

Destoumieux D, Bulet P, Strub JM, Van Dorsselaer A, and Bachere E. 1999. Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur J Biochem. 266: 335–346.

Destoumieux D, Munoz M, Cosseau C, Rodriguez J, Bulet P, Comps M, and Bachere E. 2000. Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. J Cell Sci. 113: 461–469.

Dong B and Xiang JH. 2007. Discovery of genes involved in defense/immunity functions in a haemocytes cDNA library from *Fenneropenaeus chinensis* by ESTs annotation. Aquaculture. 272: 208–215.

Douglas SE, Gallant JW, Gong Z, and Hew C. 2001. Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum). Dev Comp Immunol. 25: 137–147.

Douglas SE, Gallant JW, Liebscher RS, Dacanay A, and Tsoi SCM. 2003a. Identification and expression analysis of hepcidin–like antimicrobial peptides in bony fish. Dev Comp Immunol. 27: 589–601.

Douglas SE, Patrzykat A, Pytyck J, and Gallant JW. 2003b. Identification, structure and differential expression of novel pleurocidins clustered on the genome of the winter flounder, *Pseudopleuronectes americanus* (Walbaum). Eur J Biochem. 270: 3720–3730.

Dunham RA, Warr GW, Nichols A, Duncan PL, Argue B, Middleton D, and Kucuktas H. 2002. Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. Mar Biotechnol. 4: 338–344.

Dupuy JW, Bonami JR, and Roch P. 2004. A synthetic antibacterial peptide from *Mytilus galloprovincialis* reduces mortality due to white spot syndrome virus in palaemonid shrimp. J Fish Dis. 27: 57–64.

Eckert R, He J, Yarbrough DK, Qi F, Anderson MH, and Shi W. 2006. Targeted killing of *Streptococcus mutans* by a pheromone-guided "smart" antimicrobial peptide. Antimicrob Ag Chemother. 50: 3651–3657.

Falco A, Brocal I, Perez L, Coll JM, Estepa A, and Tafalla C. 2008a. In vivo modulation of the rainbow trout (*Oncorhynchus mykiss*) immune response by the human alpha defensin 1, HNP1. Fish Shellfish Immunol. 24: 102–112.

Falco A, Chico V, Marroqui L, Perez L, Coll JM, and Estepa A. 2008b. Expression and antiviral activity of a beta-defensin-like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences. Mol Immunol. 45: 757–765.

Falco A, Mas V, Tafalla C, Perez L, Coll JM, and Estepa A. 2007. Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. Antiviral Res. 76: 111–123.

Fedders H, Michalek M, Grotzinger J, and Leippe M. 2008. An exceptional salt-tolerant antimicrobial peptide derived from a novel gene family of haemocytes of the marine invertebrate *Ciona intestinalis*. Biochem J. 416: 65–75.

Fernandes JM and Smith VJ. 2002. A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. Biochem Biophys Res Commun. 296: 167–171.

Fernandes JMO, Saint N, Kemp GD, and Smith VJ. 2003. Oncorhyncin III: a potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, Oncorhynchus mykiss. Biochem J. 373: 621–628.

Freidinger RM. 2003. Design and synthesis of novel bioactive peptides and peptidomimetics. J Med Chem. 46: 5553–5566.

Fujii N and Tamamura H. 2001. Peptide-lead CXCR4 antagonists with high anti-HIV activity. Curr Opin Investig Drugs. 2: 1198–1202.

Gestal C, Costa M, Figueras A, and Novoa B. 2007. Analysis of differentially expressed genes in response to bacterial stimulation in hemocytes of the carpet-shell clam *Ruditapes decussatus*: identification of new antimicrobial peptides. Gene 406: 134–143.

Gomez G-D and Balcazar J-L. 2008. A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol Med Microbiol. 52: 145–154.

Greenshields AL, Knickle LC, Syvitski R, and Douglas SE. 2008. Strategies for recombinant expression of small, highly disulphide-bonded, cationic antimicrobial peptides. Protein Pept Lett. 15: 985–994.

Gross PS, Bartlett TC, Browdy CL, Chapman RW, and Warr GW. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp,

Litopenaeus vannamei, and the Atlantic White Shrimp, *L. setiferus*. Dev Comp Immunol. 25: 565–577.

Gueguen Y, Cadoret JP, Flament D, Barreau-Roumiguiere C, Girardot AL, Garnier J, Hoareau A, Bachere E, and Escoubas JM. 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene. 303: 139–145.

Gueguen Y, Garnier J, Robert L, Lefranc MP, Mougenot I, de Lorgeril J, Janech M, Gross PS, Warr GW, Cuthbertson B, Barracco MA, Bulet P, Aumelas A, Yang Y, Bo D, Xiang J, Tassanakajon A, Piquemal D, and Bachere E. 2006a. PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature. Dev Comp Immunol. 30: 283–288.

Gueguen Y, Herpin A, Aumelas A, Garnier J, Fievet J, Escoubas JM, Bulet P, Gonzalez M, Lelong C, Favrel P, and Bachere E. 2006b. Characterization of a defensin from the oyster *Crassostrea gigas*. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. J Biol Chem. 281: 313–323.

Hancock RE and Sahl HG. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 24: 1551–1557.

Iijima N, Tanimoto N, Emoto Y, Morita Y, Uematsu K, Murakami T, and Nakai T. 2003. Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream. Chrysophrys major Eur J Biochem. 270: 675–686.

Ingham AB and Moore RJ. 2007. Recombinant production of antimicrobial peptides in heterologous microbial systems. Biotechnol Appl Biochem. 47: 1–9.

Jang WS, Kim CH, Kim KN, Park SY, Lee JH, Son SM, and Lee IH. 2003. Biological activities of synthetic analogs of halocidin, an antimicrobial peptide from the tunicate *Halocynthia aurantium*. Antimicrob Ag Chemother. 47: 2481–2486.

Jang WS, Kim HK, Lee KY, Kim SA, Han YS, and Lee IH. 2006. Antifungal activity of synthetic peptide derived from halocidin, antimicrobial peptide from the tunicate, *Halocynthia aurantium*. FEBS Lett. 580: 1490–1496.

Jang WS, Kim KN, Lee YS, Nam MH, and Lee IH. 2002. Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. FEBS Lett. 521: 81–86.

Jeney G and Anderson DP. 1993. Glucan injection or bath exposure given alone or in combination with a bacterin enhance the non-specific defense mechanisms in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 116: 29–35.

Jenssen H, Hamill P, and Hancock RE. 2006. Peptide antimicrobial agents. Clin Microbiol Rev. 19: 491–511.

Jia X, Patrzykat A, Devlin RH, Ackerman PA, Iwama GK, and Hancock RE. 2000. Antimicrobial peptides protect coho salmon from *Vibrio anguillarum* infections. Appl Environ Microbiol. 66: 1928–1932.

Jiravanichpaisal P, Lee SY, Kim YA, Andren T, and Soderhall I. 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. Dev Comp Immunol. 31: 441–455.

Jung HJ, Park Y, Sung WS, Suh BK, Lee J, Hahm KS, and Lee DG. 2007. Fungicidal effect of pleurocidin by membrane-active mechanism and design of enantiomeric analogue for proteolytic resistance. Biochim Biophys Acta—Biomembranes. 1768: 1400–1405.

Kawabata S, Nagayama R, Hirata M, Shigenaga T, Agarwala KL, Saito T, Cho J, Nakajima H, Takagi T, and Iwanaga S. 1996. Tachycitin, a small granular component in horseshoe crab hemocytes, is an antimicrobial protein with chitin-binding activity. J Biochem. 120: 1253–1260.

Khoo L, Robinette DW, and Noga EJ. 1999. Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes. Mar Biotechnol. 1: 44–51.

Kjuul AK, Bullesbach EE, Espelid S, Dunham R, Jorgensen TO, Warr GW, and Styrvold OB. 1999. Effects of cecropin peptides on bacteria pathogenic to fish. J Fish Dis. 22: 387–394.

Kornbluth RS and Stone GW. 2006. Immunostimulatory combinations: designing the next generation of vaccine adjuvants. J Leukoc Biol. 80: 1084–1102.

Kraus D and Peschel A. 2006. Molecular mechanisms of bacterial resistance to antimicrobial peptides. Curr Topics Microbiol Immunol. 306: 231–250.

Kumar R, Mukherjee SC, Ranjan R, and Nayak SK. 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. Fish Shellfish Immunol. 24: 168–172.

Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, and Yang D. 2005. Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide

receptor-like 2 as the receptor and acts as an immune adjuvant. J Immunol. 174: 6257–6265.

Laing KJ, Zou JJ, Purcell MK, Phillips R, Secombes CJ, and Hansen JD. 2006. Evolution of the CD4 family: teleost fish possess two divergent forms of CD4 in addition to lymphocyte activation gene-3. J Immunol. 177: 3939–3951.

Lauth X, Shike H, Burns JC, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Nizet V, Taylor SW, Shimizu C, and Bulet P. 2002. Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass. J Biol Chem. 277: 5030–5039.

Lazarovici P, Primor N, and Loew LM. 1986. Purification and pore-forming activity of two hydrophobic polypeptides from the secretion of the Red Sea Moses sole (*Pardachirus marmoratus*). J Biol Chem. 261: 16704–16713.

Lee IH, Cho Y, and Lehrer RI. 1997a. Styelins, broad-spectrum antimicrobial peptides from the solitary tunicate, *Styela clava*. Comp Biochem Physiol B Biochem Mol Biol. 118: 515–521.

Lee IH, Lee YS, Kim CH, Kim CR, Hong T, Menzel L, Boo LM, Pohl J, Sherman MA, Waring A, and Lehrer RI. 2001a. Dicynthaurin: an antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. Biochim Biophys Acta. 1527: 141–148.

Lee IH, Zhao C, Cho Y, Harwig SS, Cooper EL, and Lehrer RI. 1997b. Clavanins, α-helical antimicrobial peptides from tunicate hemocytes. FEBS Lett. 400: 158–162.

Lee IH, Zhao C, Nguyen T, Menzel L, Waring AJ, Sherman MA, and Lehrer RI. 2001b. Clavaspirin, an antibacterial and haemolytic peptide from *Styela clava*. J Pept Res. 58: 445–456.

Li C, Haug T, Styrvold OB, Jorgensen TO, and Stensvag K. 2008. Strongylocins, novel antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*. Dev Comp Immunol. 32: 1430–1440.

Li C, Song L, Zhao J, Zhu L, Zou H, Zhang H, Wang H, and Cai Z. 2007. Preliminary study on a potential antibacterial peptide derived from histone H2A in hemocytes of scallop *Chlamys farreri*. Fish Shellfish Immunol. 22: 663–672.

Li L, Wang JX, Zhao XF, Kang CJ, Liu N, Xiang JH, Li FH, Sueda S, and Kondo H. 2005. High level expression, purification, and characterization of the shrimp antimicrobial peptide, ch-penaeidin, in *Pichia pastoris*. Protein Expr Purif. 39: 144–151.

Mar Costa M, Novoa B, and Figueras A. 2008. Influence of beta-glucans on the immune responses of carpet shell clam (*Ruditapes decussatus*) and Mediterranean mussel (*Mytilus galloprovincialis*). Fish Shellfish Immunol. 24: 498–505.

Mitta G, Hubert F, Noel T, and Roch P. 1999a. Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. Eur J Biochem. 265: 71–78.

Mitta G, Vandenbulcke F, Hubert F, and Roch P. 1999b. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J Cell Sci. 112: 4233–4242.

Mitta G, Vandenbulcke F, Hubert F, Salzet M, and Roch P. 2000a. Involvement of mytilins in mussel antimicrobial defense. J Biol Chem. 275: 12954–12962.

Mitta G, Vandenbulcke F, Noel T, Romestand B, Beauvillain JC, Salzet M, and Roch P. 2000b. Differential distribution and defence involvement of antimicrobial peptides in mussel. J Cell Sci. 113: 2759–2769.

Mitta G, Vandenbulcke F, and Roch P. 2000c. Original involvement of antimicrobial peptides in mussel innate immunity. FEBS Lett. 486: 185–190.

Miyata T, Tokunaga F, Yoneya T, Yoshikawa K, Iwanaga S, Niwa M, Takao T, and Shimonishi Y. 1989. Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II, and polyphemusins I and II: chemical structures and biological activity. J Biochem. 106: 663–668.

Morimoto M, Mori H, Otake T, Ueba N, Kunita N, Niwa M, Murakami T, and Iwanaga S. 1991. Inhibitory effect of tachyplesin I on the proliferation of human immunodeficiency virus *in vitro*. Chemother. 37: 206–211.

Morvan A, Iwanaga S, Comps M, and Bachere E. 1997. *In vitro* activity of the *Limulus* antimicrobial peptide tachyplesin I on marine bivalve pathogens. J Invertebr Pathol. 69: 177–182.

Mulero I, Noga EJ, Meseguer J, Garcia-Ayala A, and Mulero V. 2008a. The antimicrobial peptides piscidins are stored in the granules of professional phagocytic granulocytes of fish and are delivered to the bacteria-containing phagosome upon phagocytosis. Dev Comp Immunol. 32: 1531–1538.

Mulero I, Sepulcre MP, Fuentes I, Garcia-Alcazar A, Meseguer J, Garcia-Ayala A, and Mulero V. 2008b. Vaccination of larvae of the bony fish gilthead seabream reveals a lack of correlation between lymphocyte development and adaptive immunocompetence. Mol Immunol. 45: 2981–2989.

Munoz M, Vandenbulcke F, Garnier J, Gueguen Y, Bulet P, Saulnier D, and Bachere E. 2004. Involvement of penaeidins in defense reactions of the shrimp

Litopenaeus stylirostris to a pathogenic vibrio. Cell Mol Life Sci. 61: 961–972.

Munoz M, Vandenbulcke F, Gueguen Y, and Bachere E. 2003. Expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *Penaeus vannamei*. Dev Comp Immunol. 27: 283–289.

Munoz M, Vandenbulcke F, Saulnier D, and Bachere E. 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. Eur J Biochem. 269: 2678–2689.

Murakami T, Niwa M, Tokunaga F, Miyata T, and Iwanaga S. 1991. Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. Chemother. 37: 327–334.

Murray HM, Gallant JW, and Douglas SE. 2003. Cellular localization of pleurocidin gene expression and synthesis in winter flounder gill using immunohistochemistry and *in situ* hybridization. Cell Tissue Res. 312: 197–202.

Murray HM, Leggiadro CT, and Douglas SE. 2007. Immunocytochemical localization of pleurocidin to the cytoplasmic granules of eosinophilic granular cells from the winter flounder gill. J Fish Biol. 70: 336–345.

Nakamura T, Furunaka H, Miyat T, Tokunaga F, Muta T, and Iwanaga S. 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachyplesus tridentatus*). J Biol Chem. 263: 16709–16713.

Noga EJ, Silphaduang U, Park NG, Seo JK, Stephenson J, and Kozlowicz S. 2009. Piscidin 4, a novel member of the piscidin family of antimicrobial peptides. Comp Biochem Physiol B. 152(4): 299–305.

Pallavicini A, Mar Costa M, Gestal C, Dreos R, Figueras A, Venier P, and Novoa B. 2008. High sequence variability of myticin transcripts in hemocytes of immune-stimulated mussels suggests ancient host–pathogen interactions. Dev Comp Immunol. 32: 213–226.

Pan CY, Chen JY, Cheng YS, Chen CY, Ni IH, Sheen JF, Pan YL, and Kuo CM. 2007. Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*), and its role in protecting fish against pathogenic infection. DNA Cell Biol. 26: 403–413.

Park CB, Lee JH, Park IY, Kim MS, and Kim SC. 1997. A novel antimicrobial peptide from the loach, *Misgurnus anguillicaudatus*. FEBS Lett. 411: 173–178.

Park IY, Park CB, Kim MS, and Kim SC. 1998. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Lett. 437: 258–262.

Patrzykat A, Gallant JW, Seo JK, Pytyck J, and Douglas SE. 2003. Novel antimicrobial peptides derived from flatfish genes. Antimicrob Ag Chemother. 47: 2464–2470.

Powers JP and Hancock RE. 2003. The relationship between peptide structure and antibacterial activity. Peptides. 24: 1681–1691.

Raa J. 1996. The use of immunostimulatory substances in fish and shellfish farming. Rev Fish Sci. 4: 229–288.

Rajesh Kumar S, Ishaq Ahmed VP, Parameswaran V, Sudhakaran R, Sarath Babu V, and Sahul Hameed AS. 2008. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio (Listonella) anguillarum*. Fish Shellfish Immunol. 25: 47–56.

Reddy KV, Yedery RD, and Aranha C. 2004. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents. 24: 536–547.

Ren HL, Wang KJ, Zhou HL, and Yang M. 2006. Cloning and organisation analysis of a hepcidin-like gene and cDNA from Japan sea bass, *Lateolabrax japonicus*. Fish Shellfish Immunol. 21: 221–227.

Richards RC, O'Neil DB, Thibault P, and Ewart KV. 2001. Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*). Biochem Biophys Res Commun. 284: 549–555.

Robinette D, Wada S, Arroll T, Levy MG, Miller WL, and Noga EJ. 1998. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. Cell Mol Life Sci. 54: 467–475.

Roch P, Beschin A, and Bernard E. 2004. Antiprotozoan and antiviral activities of non-cytotoxic truncated and variant analogues of mussel defensin. Evid Based Complement Alternat Med. 1: 167–174.

Roch P, Yang Y, Toubiana M, and Aumelas A. 2008. NMR structure of mussel mytilin, and antiviral-antibacterial activities of derived synthetic peptides. Dev Comp Immunol. 32: 227–238.

Rocha A, Ruiz S, Estepa A, and Coll JM. 2003. Fish as biofactories: inducible genetic systems and gene targeting. Span J Agric Res. 1: 3–11.

Romestand B, Molina F, Richard V, Roch P, and Granier C. 2003. Key role of the loop connecting the two beta strands of mussel defensin in its antimicrobial activity. Eur J Biochem. 270: 2805–2813.

Saito T, Kawabata S, Shigenaga T, Takayenoki Y, Cho J, Nakajima H, Hirata M, and Iwanaga S. 1995. A novel big defensin identified in horseshoe crab

hemocytes: isolation, amino acid sequence, and antibacterial activity. J Biochem. 117: 1131–1137.

Salinas I, Abelli L, Bertoni F, Picchietti S, Roque A, Furones D, Cuesta A, Meseguer J, and Esteban MA. 2008. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 25: 114–123.

Sallum UW and Chen TT. 2008. Inducible resistance of fish bacterial pathogens to the antimicrobial peptide cecropin B. Antimicrob Ag Chemother. 52: 3006–3012.

Sarmasik A and Chen TT. 2003. Bactericidal activity of cecropin B and cecropin P1 expressed in fish cells (CHSE-214): application in controlling fish bacterial pathogens. Aquaculture. 220: 183–194.

Sarmasik A, Warr G, and Chen TT. 2002. Production of transgenic medaka with increased resistance to bacterial pathogens. Mar Biotechnol. 4: 310–322.

Schnapp D, Kemp GD, and Smith VJ. 1996. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur J Biochem. 240: 532–539.

Secombes C. 2008. Will advances in fish immunology change vaccination strategies? Fish Shellfish Immunol. 25: 409–416.

Shai Y, Fox J, Caratsch C, Shih Y-L, Edwards C, and Lazarovici P. 1988. Sequencing and synthesis of pardaxin, a polypeptide from the Red Sea Moses sole with ionophore activity. FEBS Lett. 242: 161–166.

Shen YQ, Xiang JH, Wang B, Li FH, and Tong W. 2004. Discovery of immune related factors in *Fenneropenaeus chinensis* by annotation of ESTs. Progr Nat Sci. 14: 47–54.

Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Shimizu C, and Burns JC. 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur J Biochem. 269: 2232–2237.

Silphaduang U, Colorni A, and Noga EJ. 2006. Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Dis Aq Org. 72: 241–252.

Silphaduang U and Noga EJ. 2001. Peptide antibiotics in mast cells of fish. Nature. 414: 268–269.

Sirtori LR, Cladera-Olivera F, Lorenzini DM, Tsai SM, and Brandelli A. 2006. Purification and partial characterization of an antimicrobial peptide produced by *Bacillus* sp. strain P45, a bacterium from the Amazon basin fish *Piaractus mesopotamicus*. J Gen Appl Microbiol. 52: 357–363.

Smith VJ, Fernandes JM, Kemp GD, and Hauton C. 2008. Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans. Dev Comp Immunol. 32: 758–772.

Srinivasulu B, Syvitski R, Seo JK, Mattatall NR, Knickle LC, and Douglas SE. 2008. Expression, purification and structural characterization of recombinant hepcidin, an antimicrobial peptide identified in Japanese flounder, *Paralichthys olivaceus*. Protein Expr Purif. 61: 36–44.

Stensvag K, Haug T, Sperstad SV, Rekdal O, Indrevoll B, and Styrvold OB. 2008. Arasin 1, a proline-arginine-rich antimicrobial peptide isolated from the spider crab, *Hyas araneus*. Dev Comp Immunol. 32: 275–285.

Sung W-S, Lee J, and Lee D-G. 2008. Fungicidal effect of piscidin on *Candida albicans*: pore formation in lipid vesicles and activity in fungal membranes. Biol Pharm Bull. 31: 1906–1910.

Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, and Tassanakajon A. 2004. Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. Dis Aquat Org. 61: 123–135.

Supungul P, Klinbunga S, Pichyangkura R, Jitrapakdee S, Hirono I, Aoki T, and Tassanakajon A. 2002. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). Mar Biotechnol. 4: 487–494.

Tamamura H, Otaka A, Murakami T, Ishihara T, Ibuka T, Waki M, Matsumoto A, Yamamoto N, and Fujii N. 1996. Interaction of an anti-HIV peptide, T22, with gp120 and CD4. Biochem Biophys Res Commun. 219: 555–559.

Tanguy A, Bierne N, Saavedra C, Pina B, Bachere E, Kube M, Bazin E, Bonhomme F, Boudry P, Boulo V, Boutet I, Cancela L, Dossat C, Favrel P, Huvet A, Jarque S, Jollivet D, Klages S, Lapegue S, Leite R, Moal J, Moraga D, Reinhardt R, Samain JF, Zouros E, and Canario A. 2008. Increasing genomic information in bivalves through new EST collections in four species: development of new genetic markers for environmental studies and genome evolution. Gene. 408: 27–36.

Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, Sritunyalucksana K, Phongdara A, Pongsomboon S, Supungul P, Tang S, Kuphanumart K, Pichyangkura R, and Lursinsap C. 2006. *Penaeus monodon* gene discovery project: the generation of an EST

collection and establishment of a database. Gene. 384: 104–112.

Tian JY, Sun XQ, and Chen XG. 2008. Formation and oral administration of alginate microspheres loaded with pDNA coding for lymphocystis disease virus (LCDV) to Japanese flounder. Fish Shellfish Immunol. 24: 592–599.

Tincu JA and Taylor SW. 2004. Antimicrobial peptides from marine invertebrates. Antimicrob Ag Chemother. 48: 3645–3654.

Tonheim TC, Bogwald J, and Dalmo RA. 2008. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol. 25: 1–18.

Ullal AJ, Wayne Litaker R, and Noga EJ. 2008. Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev Comp Immunol. 32: 1301–1312.

Uzzell T, Stolzenberg ED, Shinnar AE, and Zasloff M. 2003. Hagfish intestinal antimicrobial peptides are ancient cathelicidins. Peptides. 24: 1655–1667.

Yang M, Wang KJ, Chen JH, Qu HD, and Li SJ. 2007. Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B.). Fish Shellfish Immunol. 23: 1060–1071.

Yeaman M and Yount NY. 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev. 55: 27–55.

Zaiou M. 2007. Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. J Mol Med. 85: 317–329.

Chapter 8

Adaptive Immunity in Finfish: A Physiological Perspective

Brian Dixon and Leandro A. Becker

INTRODUCTION

The fish immune system has always been interesting from an evolutionary biological perspective since fish evolved at the beginning of the vertebrate radiation. However, during the second half of the last century, the research on the topic has also increased exponentially with the aim of improving fish health care in order to secure aquaculture production in a growing market. Therefore, the ultimate goal of this chapter is to present the main characteristics of the fish innate and, in particular, adaptive immune systems together with current and potential mechanisms in monitoring and preventing diseases.

One characteristic of the taxonomic groups commonly known as "fish" is the enormous diversity they contain, since "fish" includes several heterogeneous lineages that are distantly related (Carroll 1988). Teleostei is the most diversified group of all vertebrates comprising about half of the extant vertebrate species (Nelson 1994). Different fish lineages immune systems are comparable at the genomic level (Stet et al. 2003; keeping in mind not all of them have completed rediploidization), but not at the functional level. For instance, rainbow trout, coho, and Atlantic salmon—all salmonid species—have histological and biochemical differences in skin and mucus (Fast et al. 2002). Rainbow trout and brook charr are both infected by the haemoflagellate *Cryptobia salmositica*, but it does not cause anemia in the brook charr even though the same or higher levels of parasite load are found in both species (Zuo and Woo 1997). Another example is the variation in humoral immune responses: while there is a strong humoral response in salmonids, it is almost nonexistent in Atlantic cod (Lund et al. 2006; Solem and Stenvik 2006). Therefore, findings in any particular species cannot be generalized to all the fish, even within taxonomic designations such as the teleostei. Thus, each species targeted for aquaculture production needs to be investigated separately to determine its immunological physiology and maximize its performance. The description of the fish immune system in this section tends to be general with some examples where necessary, and it does not intend to comprehensively cover specific peculiarities found in particular species.

THE IMMUNE SYSTEM AS A WHOLE INTEGRATIVE DEFENCE MECHANISM

Traditionally, the vertebrate immune system has been divided into two components: innate and adaptive immune systems. The difference is attributed

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

to the specificity of the response against pathogens. The first one consists of all the immune response elements present before encountering a pathogen. These raise nonspecific responses to invariant molecular patterns found in the majority of microorganisms by means of germline encoded receptors. Adaptive immunity develops only after microorganisms overwhelm the former, raising specific responses to specific molecular components (antigens) of pathogens through specific receptors generated through gene rearrangement.

However, innate and adaptive immune systems are artificial designations since they both form part of an integrative immune system in which they interact and regulate each other. The innate immune system detects the entrance of foreign antigens, initiating a series of responses that, if not successful at eliminating the infectious agent, will help to determine the type of adaptive immune response to be elicited. Once established, the antigen-driven adaptive elements target the pathogen and direct the effector elements of innate immunity to efficiently clear up the infectious agent.

Main Features of the Fish Innate Immune System

The innate immune system recognizes conserved molecular patterns that are essential products of microbial physiology. They are collectively designated pathogen-associated molecular patterns (PAMP) and include polysaccharides, lipopolysaccharide, peptidoglycans, unmethylated CpG motifs from bacterial DNA, double-stranded viral RNA, and other molecules not normally found on the surface of multicellular organisms (Medzhitov and Janeway 2002). These are recognized by germline-encoded receptors designated pattern recognition receptors (PRR; Dixon and Stet 2001), which are strategically expressed on cells that are the first to encounter pathogens during infection, such as surface epithelia and effector cells of the innate immune system, including antigen-presenting cells (APC; Medzhitov and Janeway 1997). Genes encoding several of these PRRs have now been isolated from many different species of fish, including at least 17 toll-like receptors from at least 12 fish species (Rebl et al. 2010).

Humoral Innate Immunity

Skin and scales, together with gill and gut epithelia, are the physical barrier to infectious organisms. The mucus secreted by fish epithelial cells is an effective nonspecific defense; its viscous consistency helps to trap microorganisms (Shephard 1994). It possesses several humoral factors such as antimicrobial peptides, lectins, pentraxins, lysozyme, complement proteins, and natural antibodies, which together inhibit the growth of and destroy bacteria (Alexander and Ingram 1992; Fast et al. 2002). If pathogens are successful in breaching this initial line of defense and are able to establish a focus of infection, they will next face the same and other humoral factors found in serum plus the cellular components of the innate immune system. Among the growth inhibitors present in serum are hepcidin and transferrin—chelators of the available iron essential for the bacteria (Ellis 2001; Rodrigues et al. 2006)—and interferon-like molecules that induce the expression of Mx and other antiviral proteins (Leong et al. 1998; Ellis 2001).

Natural antibodies are immunoglobulins (Igs) present in the body fluids of healthy individuals prior to an infection (Sinyakov et al. 2002). While Igs are an adaptive immune component, natural antibodies are produced in the absence of antigen exposure and thus they are considered part of the innate system. They are polyreactive and have a low affinity, but a broad specificity for both self epitopes and nonself PAMPs (Avrameas and Ternynck 1995). The tetrameric configuration of IgM in fish (pentameric in tetrapods) helps counteract the low affinity and allows them to activate complement through Fc receptors, which mediate the interaction of antibody–antigen complexes with cells, resulting in a variety of immune responses, mainly phagocytosis (Boes 2000). There is evidence of natural antibody activity in fish, especially in cod and other gadids, where natural antibodies seem to comprise the majority of their circulating Igs (Gudmundsdottir et al. 2009).

Complement proteins in fish are more diverse than their mammalian counterparts. For instance, C3 seems to be coded by several genes in some species and these genes show some degree of polymorphism in trout and carp (Sunyer and Lambris 1998; Zarkadis et al. 2001). Considering that pathogens

can be opsonized by proteins of the complement system enhancing phagocytosis and lysis (Boshra et al. 2005, 2006), a combination of polymorphism and gene duplication could generate a large C3 repertoire, where different isoforms of C3 could confer resistance to different pathogens (Sunyer et al. 1998). Many genes in tetraploid fish have become pseudogenes, have been diploidized, or their products have gained new functions (Allendorf and Thoorgard 1984). Therefore, the maintenance of these duplicated C3 genes must have important functional implications (Sunyer et al. 1998). The diversity of the fish complement system together with its higher activity and lower temperature activation compared with their mammalian counterparts (Sakai 1992) suggest it is a critical factor for fish survival (Sunyer et al. 1998).

Cellular Innate Immunity

Teleosts develop a cellular innate response with leukocytes that are morphologically and functionally equivalent to mammalian macrophages, neutrophils, monocytes, thrombocytes, natural killer cells, and eosinophils (Hine 1992; Afonso et al. 1997; Miller et al. 1998; Ellis 1999). Teleost B cells that produce natural antibodies functionally resemble those of mammalian B-1 cells, expressing membrane IgM as a monomer and secreting soluble IgM as a tetramer (Li et al. 2006; although some species seem to secrete redox versions of IgM, see Kaattari et al. 1998).

Pathogens are targeted by phagocytic cells, mainly macrophages, granulocytes, and dendritic cells through scavenger, Fc, and complement receptors (Zapata and Amemiya 2000), and also by phagocytic B cells (Li et al. 2006). After phagocytosis, secretion of factors and cytokines by these particular cells lead to inflammation in the site and recruitment of other leukocytes, such as neutrophils, while APCs process captured foreign particles and present peptide fragments to T cells, which afterward are activated and initiate the appropriate adaptive immune response. While many of the main components of innate immunity have been found in teleost fish and are presumed to function in a manner analogous to their tetrapod, particularly mammalian counterparts, many details regarding the function of these molecules and the manner in which they interact or induce adaptive responses have not been examined in detail in teleosts.

Main Features of the Fish Adaptive Immune System

The adaptive immune system evolved about 500 mya in an ancestor of the jawed vertebrates (Cannon et al. 2004). All extant classes of jawed vertebrates possess Igs, T-cell antigen receptors (TCRs), major histocompatibility (MH) genes, and the recombination-activating genes (RAG; Cannon et al. 2004). It is the receptor gene rearrangement mediated by RAG, a defining characteristic of lymphocytes, that makes the specificity of the vertebrate's adaptive immune system possible by allowing the generation of a near-infinite variety of receptors, each expressed by a different individual T cell or B cell (Jones and Simkus 2009). The generation of TCR diversity in the thymus is similar in almost all jawed vertebrate taxa, whereas BCR diversity is generated in different ways and in different primary lymphoid tissues (Flajnik 2002). Besides the RAG-mediated somatic recombination, all jawed vertebrate taxa have evolved different mechanisms to further increase their somatic variation of Igs in B cells, such as class switching, gene conversion, and somatic hypermutation (Flajnik 2002), where the same set of enzymes govern all these mechanisms (Muramatsu et al. 2000; Arakawa et al. 2002; Barreto et al. 2005). Although bony fish possess these enzymes, they exhibit only somatic hypermutation (Yang et al. 2006), as do sharks (Malacek et al. 2005). Bony fish express IgM and IgD in mature naïve B cells and activated B cells lacking a secondary diversification of antibody repertoire through DNA recombination as in mammals (Wilson et al. 1997). The absence of class switching seems to be due to a lack of switch regions (repetitive DNA sequences) and multiple constant (C) region genes in the Ig heavy (IgH) chain locus (Hordvik 1998; Stavnezer and Amemiya 2004; Barreto et al. 2005). It is important to note that while there is a unique isotype of immunoglobulin in teleosts, IgT, its constant gene is upstream of the IgM C gene and there is no evidence to date of class switching between these isotypes (Hansen et al. 2005). The ability to express different C heavy isotypes evolved early, as even the most primitive jawed vertebrates, cartilaginous fish (e.g.,

sharks), have multiple C heavy genes (Stavnezer and Amemiya 2004). Kaattari et al. (1998) described different redox forms of IgM in teleosts, proposing each have a different function, somehow substituting for the lack of Ig isotype diversity in teleosts.

The fish Ig light (IgL) chain is comparable to other vertebrates in terms of the number and diversity of their IgV regions (Warr 1995). Teleost fish IgH loci have a typical translocon arrangement and, although their IgL loci have a multicluster arrangement, which would favor rearrangement of elements within each cluster, there is no evidence for a restriction in the generation of combinatorial and junctional diversity in fish (Warr 1995; Hsu 1998). However, antibodies of ectothermic vertebrates are of much lower affinity than those of latest vertebrates such as birds and mammals (du Pasquier 1982; Wilson and Warr 1992; Warr 1995), and increases in avidity were seen to occur late in the immune response of trout (Kaattari et al. 2002).

One of the elusive questions about the adaptive immune response in fish is to what extent they can develop immunological memory since there are contradictory reports about the increase in antibody titers after subsequent immunizations. The differences seem to be related, at least in part, to the amount and nature of antigen administered. Secondary immune responses were shown to occur in rainbow trout when a complex antigen, formalin-inactivated bacteria, was used for immunization but failed when using DNP-hapten antigen (Cossarini-Dunier 1986). Antigenic determinants are much more numerous on the bacterin than in DNP hapten, and thus more clones may have been recruited against the former (Cossarini-Dunier 1986).

By definition, a memory immune response occurs when a host is repeatedly exposed to the same pathogen and a faster and stronger response occurs each time (Tangye and Tarlinton 2009). Memory responses in mammals are characterized by small amounts of IgM and highly developed affinity maturation with large amounts of IgG. Recent studies indicate that the presence of small numbers of antigen-specific B cells with IgG receptors is sufficient for the heightened antibody response in mammals (Martin and Goodnow 2002).

Conversely, fish Ig isotypes do not switch from IgM and IgD to IgT, their affinity maturation is rather poor, and when it is seen, it takes several weeks to develop (Kaattari et al. 2002). An interesting feature of multivalent antibodies, as the case of the pentameric IgM in mammals, is that bearing the same intrinsic affinity as an IgG antibody, their extrinsic avidity is logarithmically greater (Hornick and Karush 1972). The same has been shown in monomeric and pentameric IgMs of the nurse shark (Voss and Sigel 1972). Considering that fish only secrete the tetrameric form of IgM, its avidity for pathogens might be good enough to opsonize them and thus it is plausible that fish can simply rely on the initial antibody diversity from which those clones bearing the highest affinities for the antigen are exponentially expanded (Kaattari et al. 2002). Memory seems to be, at least in trout, due to a simple expansion of the antigen-specific precursor pool without many of the qualitative changes in antibody or B-cell function associated with memory in mammals (Arkoosh and Kaattari 1991). It is thought that, although fish do not possess germinal centers, there is a mechanism for antigen-driven clonal selection that could be responsible for the late affinity maturation (Kaattari et al. 2002). More studies are needed to confirm this though.

Thus, although all the mechanisms of the humoral branch of adaptive immune system are present in fishes and other groups of early vertebrates, they seem to work at a more basal level, lacking the more sophisticated features found in mammals, such as a high level of affinity maturation, isotype switching, and an effective memory. Many hypotheses have been proposed for this, even considering nonimmune elements such as cell cycle properties, mode of embryonic development, and body temperature (du Pasquier 1982). It has also been suggested that early vertebrates can still rely more on innate defense mechanisms that they inherited from invertebrates, such as natural haemolysin and complement, which recognize bacterial surfaces, lyse these cells, and prevent the adaptive immune system of the animals from seeing many antigens and by doing so, reducing the pressure for diversification of antibody repertoire (du Pasquier 1982). This hypothesis suggests that elimination of pathogens in early stages of infection (for instance, protective immunity by natural antibodies) could be very important in early vertebrates (Hsu 1998). The recently described

phagocytic function of the fish B cells (Li et al. 2006) might be in accordance with this view, helping to produce a faster response since T cells recognizing peptides presented by the MH receptor on the B cell will be activated immediately and can rapidly instruct the presenting B cell to secrete antibodies.

Regardless of their relatively simple adaptive immune response, many species of bony fish can generate a specific response. It is this feature, and the presumption that all of the individual components work in much the same way as they do in mammals, that is relied on when protective tools are generated, as discussed in the vaccine section below. Aquaculture depends on these measures in order to culture large quantities of fish.

MH RECEPTORS

An important characteristic of the adaptive immune system is the presentation of specific foreign particles by MH receptors, which are members of the immunoglobulin superfamily proteins and interact with T cells through a specific T cell receptor in order to initiate immune responses.

In tetrapods and sharks, they are referred to as MH complex (MHC) because their genes are linked in a complex on a single chromosome, but in teleost fish, these genes are unlinked and spread across several chromosomes, therefore the term "complex" is omitted (Shand and Dixon 2001). MH receptors are not encoded by rearranging genes but instead are highly polymorphic (Dixon et al. 1995). There are two types of MH receptors. Class I MH receptors are composed of two noncovalently linked polypeptides encoded by different genes: a "heavy" chain with three extracellular immunoglobin domains, a transmembrane domain, and a cytoplasmic domain; and a small polypeptide composed of a single immunoglobulin domain called B2-microglobulin. Class II MH receptors comprise two noncovalently linked polypeptides chains called alpha and beta, which consist of two extracellular domains, one transmembrane domain and a cytoplasmic tail. Both classes form a groove in the two outermost domains, which is used to carry peptides from pathogens out to the cell surface for presentation to T cells (Stet et al. 1996). The presentation of these foreign peptides is one of the key events in T cell activation. To complete the activation of T cells, costimulators are needed, called B7 molecules, which are detected by CD28 receptors in the T cell (Medzhitov and Janeway 2000).

The most outstanding fact about MH receptors is the high polymorphism found in natural populations, making it the most polymorphic of all known nuclear encoded genes among vertebrates (Stet et al. 2003). Balancing selection, through overdominance (heterozygote advantage) and the negative frequency-dependent selection hypotheses, is the widely accepted explanation for the high MH polymorphism (for a thorough discussion, see Bernatchez and Landry 2003). Different studies have shown that putative alleles can lead to either greater susceptibility or resistance for particular diseases (Langefors et al. 2001; Lohm et al. 2002), due to each different allele's varied capacity to bind peptides from a pathogen. Identifying resistant alleles in a species for a particular disease that affects a particular geographic area could help to improve hatchery fish stocks by selecting those families that carry them with higher frequencies of the resistance allele. The identification of those alleles can nowadays be done relatively easy through molecular cloning and DNA sequencing or by electrophoretic methods such as denaturing gradient gel electrophoresis or single strand conformation polymorphism.

ANTIGEN PRESENTATION IN THE ADAPTIVE IMMUNE RESPONSE

When pathogens succeed in surviving the innate immune system, they have to be captured and processed by APCs in order to be presented as foreign peptide fragments bound to MH molecules to T lymphocytes. After recognition of the peptide/MH molecule complex by a specific T-cell receptor, an intercommunication is developed between the APC and the T cell, where many different cytokines are upregulated in both cells. The result is the clonal expansion of B and T lymphocytes, which will respond exponentially.

It is the ability of lymphocytes to expand clonally paired with their capacity to rearrange the genes for some of their immunoglobulin superfamily member receptors that makes the adaptive immune response so strong. There are several variations in adaptive immune responses, including cell-mediated and humoral responses. Cellular immune response

generally refers to the cytotoxic activity by CD8+ T cells targeting intercellular pathogens, whereas humoral immune response involves the production of antibodies by B cells and the activation of infected macrophages to clear out extracellular and vesicular pathogens. Intracellular pathogens present in the cytoplasm are presented by MH class I molecules to CD8+ T cells, while pathogens in intracellular vesicles or phagocyted from the extracellular media are carried to the cell surface by MH class II molecules and presented to CD4+ T cells (Dixon et al. 1995).

Once activated, CD4+ T cells activate macrophages to eliminate pathogens and B cells to become immunoglobulin secretory plasma cells, thus enhancing phagocytosis through opsonization and contributing to pathogen clearance.

Activated T cells also produce a range of cytokines and chemokines that not only activate infected macrophages but also recruit fresh macrophages to sites of infection. Thus, CD4+ T cells control and coordinate host defense against both intracellular and extracellular pathogens that resist killing after being engulfed by macrophages (Sher and Kaufman 1992). CD8+ T cells are essential against pathogens that live in the cytosol, mainly viruses and some bacteria. These cytotoxic T cells kill any cell-presenting foreign peptides trough MHC class I (Sher and Kaufman 1992).

MH SEQUENCES AND THEIR APPLICATIONS

Purifying natural selection works to eliminate deleterious mutations in most of the coding sequence of genomes, as synonymous substitutions (those that do not change the amino acid in the encoded protein) usually outnumber nonsynonymous ones (Hughes and Nei 1988). Positive selection, on the other hand, favors alleles that confer an advantage to the individual, so part of the high polymorphism seen in MH genes is probably maintained because some new alleles that arise confers enhanced capability to combat a pathogen. Thus the variability, seen in the peptide-binding region of the MH molecules, can be explained because of the function of these proteins, which is to present foreign peptides to T cells.

A search for MH gene sequences in Internet databases shows the magnitude of work that has been done on this aspect of teleost adaptive immunity so far. Many MH alleles associated with disease resistance have been found in several studies involving different species (Langefors et al. 2001; Lohm et al. 2002).

MH sequences have also been used to measure immune response of different fish species (Wang et al. 2008). Research in this area continues; for example, our laboratory is involved in a project to screen MH alleles in several rainbow trout hatcheries in British Columbia with the aim of creating a database of rainbow trout MH alleles that can be associated with specific diseases, as well as another to characterize the MH alleles of walleye populations in the Great Lakes (Canada) in order to assess whether or not some populations possess alleles that will confer resistance to the recent VHSV outbreak there. Once the MH allele typing is complete, fish containing alleles that make them susceptible to disease could be removed from the hatchery broodstock and replaced with those containing resistance alleles, or in the case of the walleye, specific MH alleles could be bred into or out of the affected populations.

CYTOKINES AND CHEMOKINES AS MEASURES OF IMMUNE RESPONSES

Knowledge of cytokines, small polypeptides secreted by one cell to pass signals to another, in teleost fish has expanded dramatically in the past 10 years, thanks mostly to genome projects uncovering teleost equivalents of mammalian cytokines (Savan and Sakai 2002). Indeed, it is now evident that most of the major cytokines that govern immune responses in mammals are present in most species of teleost fish (reviewed in Secombes 2008), including the recent revelation that both interleukin-2 (Díaz-Rosales et al. 2009) and interleukin-4 (Secombes 2009) are present, at least in the salmonids. Again, while this suggests that fish immune responses seem to be regulated in much the same way as mammalian responses, it is important to note that the sequences of these cytokines are very diverged from their mammalian counterparts, often having only 25–30% identity at the amino acid level, and that fundamental differences between the anatomy of the immune systems of teleosts and mammals (the lack of lymph nodes and germinal centers in fish, for example) suggest that the function of fish homologs

of mammalian cytokines may be different enough to warrant serious investigation before they are used for clinical applications.

Fish chemokines, small polypeptides that set up chemical gradients to attract other cells either to immune organs or to sites of injury and infection, in particular are an interesting example of how similar, yet different, teleost cytokines can be from their mammalian counterparts. The first chemokines isolated from teleosts, trout CK-1 (Dixon et al. 1998), looked fairly similar to mammalian chemokines of the CC group—those possessing two cysteine residues side by side near their amino terminus. However, the next chemokine isolated from rainbow trout, CK-2 was very atypical in that it possessed an amino terminal domain that was clearly a CC chemokine, but it also possessed a long carboxyl terminal domain that resembled a highly glycosylated mucin molecule, which was not followed by a transmembrane domain (Lei et al. 2002). While some mucin stalk bearing chemokines had been found in mammals (e.g., the CXXXC chemokines; Bazan et al. 1997), none of the mammalian CC chemokines possess this feature and all mucin stalk chemokines of mammals had a transmembrane domain, suggesting a fundamentally different mode of action than CK-2. Recent genome studies in zebrafish found over 100 chemokines in zebrafish—more than were known for all mammals—of which three possessed mucin stalks and transmembrane domains, but over one-third of the chemokines found in the zebrafish genome had mucin stalks and no transmembrane domain, just like trout CK-2 (Nomiyama et al. 2008). Clearly, this group of molecules, which for the moment appears to be unique to teleosts, is important for immune function based on the number of genes found in the zebrafish genome, yet clearly they indicate that teleost immunity may be very different from mammalian immunity, despite the apparent similarities in the molecules involved. Nomiyama et al. (2008) also reported a second group of chemokines, the CX family, which is unique to teleosts.

Thus, it appears that, as in mammals, measurements of the production of key cytokines will be extremely useful for assessing teleost health status and immune responses; this information will need to be interpreted in a teleost, and perhaps even species-specific, context to be meaningful. Additionally, as discussed below, direct measurement of the production of proteins from the genes that are known will require a large effort to produce the reagents and assays—in particular, monoclonal antibodies—required to do this in a precise way.

KNOWLEDGE OF FISH IMMUNITY AND WHAT IT MEANS FOR VACCINES

Despite the lack of sophistication of the teleost immune system as compared to mammals, fish do possess an effective adaptive immune response against their pathogens. Although it takes longer to develop than in mammals, the adaptive response is exploited in aquaculture vaccination programs against common diseases.

Vaccines are the best tool available to provide immunization against potential diseases that fish may eventually face. They boost the immune system, providing the individual with a first exposure to antigens for particular pathogens without causing a disease. In most groups of fishes, this eventually will lead to clonal expansion of antigen-specific T and B cells for the production of antibodies, which in mammals helps the individual to produce a rapid immune response the second time the individual is exposed to the pathogen. As noted above, experimental evidence for memory cells in teleosts is less than conclusive, so it may be that the benefit of vaccination in teleosts is limited to a temporary boost in immunity.

Traditionally prepared vaccines consist of killed or attenuated bacterial cells, and in a few cases viruses, which are administered to the individuals mainly by injection, although some are also administered by immersion. As noted above, these vaccines are designed on the presumption that teleost immunity works in much the same way that mammalian immunity does. The development of knowledge on the way that immunology and molecular biology actually work in teleosts will give us the opportunity to develop more efficient and low-stress vaccines. Subunit vaccines incorporate a superior level of knowledge about the particulate antigens from different pathogens that elicit an immune response. A new generation of vaccines based on recombinant technology has made it possible to develop more efficient vaccines against any kind of pathogen that becomes a potential disease risk.

Method of Delivery

Vaccinations of processed antigens can be delivered in one of the following ways: intraperitoneal and intramuscular injections of particulate antigen or DNA, submersion in a water bath containing processed antigen, and oral vaccination with antigen mixed in food pellets. The injection method is widely used due to its reliability, although it causes stress on the individuals due to manipulation. Timing of delivery is another issue with vaccination since fish need to attain a minimum size to be injected, leaving them vulnerable until that point. Immersion is a method more amenable to mass vaccination, but its disadvantages are the enormous amount of vaccine needed and its protective results are more limited than with injection. A recently described method uses a multiple puncture instrument to produce small skin lesions, while fish is immersed in a vaccine suspension (Nakanishi et al. 2002). This puncture/immersion method proved to be more effective than immersion alone since higher numbers of particulate antigens were taken up by fish and delivered to the lymphoid tissues (Nakanishi et al. 2002). A protocol for massive immunization with this technique could be developed in order to be widely commercialized.

Adjuvants—The Challenge for Effective Vaccines

Adjuvants are oil-based emulsions that delay the release of the antigens into the body fluids, extending the release of the active ingredient, in order to extend protection against the disease. For teleosts, this may be important because as noted, their memory response is limited and the slow release of the active agent in the vaccine will extend the boost in immunity provided by the vaccine.

One of the more important roles of adjuvants is to provide agents that activate the innate immune response. Traditional components of adjuvants, such as heat-killed bacteria, provide the PAMPs (described in the section of this chapter entitled "Main features of the fish immune system") which activate the PRRs. Without such agents, the immune response induced by vaccines is not as strong or effective as it could be. The challenge in teleost vaccine design is that, once again, much of the knowledge driving the development of effective adjuvant components is based on compounds and knowledge from mammalian immunology. Studies on fish toll-like receptors (Rebl et al. 2010) and other innate receptors, such as ladder lectins (Young et al. 2009), will provide key information specific to teleost innate immunity that will allow the development of adjuvant components that are more specific for teleosts, hopefully providing much more effective vaccines. In addition, the isolation of genes encoding cytokines (Secombes 2008) and other key immune system molecules (Stet et al. 2003; Daeron et al. 2008) from teleosts opens the door for the use of immuno-adjuvants—the addition of natural fish immune proteins that will boost immune responses.

Live Attenuated Bacteria as Carriers

Vaccines using attenuated live bacteria as vectors carrying recombinant antigens or DNA have been proved successfully in laboratory (see Powell et al. 2009; Wang et al. 2009 for recent examples). Recombinant vaccines have been developed using attenuated live bacteria as vectors, such as *Escherichia coli* and *Salmonella*. Cytokines have been expressed in attenuated salmonella hypothesizing that in situ delivery may enhance antibody response to the attenuated bacteria (Hahn and von Specht 2003). These authors state that *Salmonella* as a vector system has been in trouble due to a lack of protective immunity it can offer. That is why there are no commercial vaccines to date.

Better protective immunity is reached when attenuated strains of bacteria that cause the disease are administered to fish. Recently, *Vibrio anguillarum* has been used as the carrier, utilizing the secretion mechanism based on *E. coli* α-haemolysin (HlyA) transport system. The recombinant proteins were fused with the α-haemolysin secretion signal and expressed from the commonly used HlyA secretion vector pMOhly1 (Shao et al. 2005). The advantages of using an attenuated pathogen as a carrier are evident. It not only produces immunity against the secreted recombinant proteins but also elicits a good protective immunity against the pathogen since common cell-surface antigens will be detected by the fish's immune system. The danger is that even an attenuate virus can produce an excessive response

in a small number of individuals, risking anaphylactic shock and death.

qPCR: A TECHNIQUE TO ASSESS ADAPTIVE IMMUNE RESPONSE

Real-time PCR has been applied with success in different studies to diagnose the presence of pathogens in different organs of fish (Jansson et al. 2008; True et al. 2008). In mammals, quantification of cytokines and some immune receptors is usually carried out using antibodies to the target proteins, but these reagents are simply not available for teleosts. Also, given the diversity in the teleost group, reagents from one species, for example Atlantic salmon, will not cross react with other commercially important species, such as cod. Thus, all of these reagents must be developed independently for each species. Therefore, at the moment, measurements of mRNA levels using qPCR represents the best hope of understanding changes in gene expression levels and ultimately assessing immune status. While microarrays are available for some species and might be used for this application, they are not widely available, they are costly, and they are not yet as accurate as qPCR.

Suppression Subtractive Hybridization and qPCR

Suppression subtractive hybridization following disease challenges or immunostimulation has been employed successfully to clone novel immune-relevant genes in fish species of commercial value such as Atlantic salmon (Tsoi et al. 2004; Matejusová et al. 2006), carp (Fujiki et al. 1999, 2000a, b), rainbow trout (Lei et al. 2002), and turbot (Wang et al. 2008). The technique involves the hybridization of control and infected fish cDNA, where the transcripts common to both groups are excluded and those ones differentially expressed are selectively amplified by suppression PCR (Diatchenko et al. 1996). From these sequences, primers for key immune genes can be designed in order to measure immune response in infected fish using qPCR. An appropriate panel of qPCR primers can be used to assess the induction of innate, humoral, or cellular responses.

Quantitative PCR Integrating Pathogen Virulence and Immune Response

A different approach is being developed in our laboratory with the goal of analyzing the interrelationship between the virulence of *V. anguillarum* and the adaptive immune response of chinook salmon. The idea is to develop probes targeting both pathogen and host mRNAs that can be extracted at the same time from the different organs of the host. The load of bacteria can also be measured using a probe for nuclear DNA, such as bacterial ribosomal RNS or protein genes. The goal of these studies is to detect a baseline gene expression to determine the pathogen load, in conjunction with probes for virulence markers, in order to determine at which point the pathogen starts causing disease in an individual. Such knowledge could have such importance that by monitoring net pens regularly and analyzing pathogen gene expression with qPCR, the majority of the population can be controlled, despite the fact that they might be carrying a small pathogen load. The technique will be tested in different hatcheries from where samples will be taken regularly and sent to our laboratory.

ENHANCING IMMUNE RESPONSES: IMMUNOSTIMULANTS

It has been shown that some food additives used as immunostimulants may enhance the fish immune system (Anderson 1992; Secombes 1994; Robertsen 1999). They include different substances generally known collectively as biological response modifiers (BRM; Leung et al. 2006). The most common group consists of polysaccharides extracted from a variety of sources such as cell wall of yeast and fungi, from which (1-3)-β-D-glucan is the most active compound (Bohn and Be Miller 1995). Glucans have been tested in a variety of fish species, including salmonids (Robertsen et al. 1990; Engstad and Robertsen 1995) and nonsalmonid species such as sea bass (Bagni et al. 2000), sea bream (Rodriguez et al. 2004; Cuesta et al. 2005), sturgeon (Jeney and Jeney 2002), halibut (Skjermo and Bergh 2004), and turbot (Villamil et al. 2003). Another group of BRMs are nucleic acids whose effects have been evaluated more recently in largemouth bass (Kubitza et al. 1997), Atlantic salmon (Burrells et al. 2001), and turbot (Low et al. 2003). Although most

of these studies concentrated on the effects of innate factors of the immune response, BRMs may also influence the antibody response following vaccination (Verlhac et al. 1996; Verlhac et al. 1998). There have also been studies with immunostimulants on larval fish to evaluate survival rates, especially if they are increased until such time that their immune system becomes fully developed (Bricknell and Dalmo 2005). Commercial products such as MacroGard® and ProVale® (yeast-derived) and Ergosan® (seaweed-based) have recently become available for use in aquaculture. Guselle et al. (2010) have examined one of these commercial products against research-grade glucan and found evidence of reduction in Xenoma formation in rainbow trout. Gioacchini et al. (2008) also evaluated another commercial product containing alginic acid and reported effects at the cytokine gene expression level. Despite the broad experimentation with these natural substances and the potential benefits associated to them, our lack of understanding of teleost innate immunity means that their mechanisms of action and the consequences their prolonged use could have on the cultivable species are not well understood. This may explain part of the reluctance to use them in the aquaculture industry. More experiments including dosage and mode of administration need to be performed with each cultured species to assess potential undesirable effects that might arise, perhaps from chronic overstimulation of the immune system. Additionally, no study has yet addressed the potential consequences of using immunostimulants at an early (i.e., larval) stage on the development of the adult immune system. If these issues can be clarified, the future consideration of immunostimulants as a prophylactic in the fight against diseases looks very promising, especially nowadays with growing concerns about the use of antibiotics in cultured organisms.

MONITORING OF HEALTH STATUS

It is widely known that handling and crowding stress, water pollution, and steep temperature changes have negative impact on the immune system of fish. Several types of stress that affect fish endocrine systems result in suppression of immune systems, thereby decreasing a fish's ability to fight diseases (e.g., Nath et al. 2006; Castillo et al. 2009; Iwanowicz et al. 2009). Regularly monitoring the health status of fish is not an easy matter and is currently not possible for most hatcheries, especially using the novel techniques described above, which need high-level training for personnel and special equipment. As in the search for biomarkers as indicators of cellular and subcellular stress induced by the environment, the search for immunomarkers and the development of easy immunoassays in the field remains a challenge for health monitoring in aquaculture. In this context, detection of upregulation of gene expression of key cytokines could be used to anticipate the outbreak of some diseases (i.e., virus-based diseases) that are difficult and time-consuming to detect using more standard techniques such as histopathology and could potentially provide more lead time for preventative strategies.

New technologies using the properties of nucleic acids could change the way fish health is examined in a rapid, reliable, and specific manner. Real-time PCR has shown its capability to detect pathogen's DNA and RNA from a minuscule piece of host tissue. By standardization of this technique and perhaps the analysis of virulence markers, a detection threshold can be set up, which when exceeded could anticipate a disease outbreak. For instance, a series of primers targeting different common pathogens could be arranged into a "screening kit" and that be distributed among hatchery-monitoring laboratories. Technology for automating this process and making it into a handheld device exists, but it will likely be a decade or more before it could be marketed.

IMPROVING BROODSTOCK AND BREEDING PRACTICES

In order to utilize our growing knowledge of teleost immunity to generate naturally disease-resistant fish in future generations in a hatchery, different approaches can be taken. The simple one could be to allow natural selection to remove all those individuals that are genetically susceptible to the disease. This obviously has a cost and might take considerable time to rebuild the stock to a commercially viable number of individuals. Not a favorable practice, but one that ensures that fish will adapt to the rearing conditions and their local environment without the use of antibiotics. The down side of this is, of course, that the genetic backgrounds left are

more limited in diversity than the original stock and may not have the desired characteristics in other important areas such as growth rate. Additionally, the genetic backgrounds lost in the selection limits the immune repertoire of the survivors and may cause them to be susceptible to new pathogens that arise. This could be a severe problem in teleosts where the MH genes that respond to viruses and bacteria are unlinked and thus segregate independently (Grimholt et al. 2003). Thus, selecting strongly for viral resistance may provide no advantage or perhaps even a disadvantage for responses to bacterial pathogens.

Another approach that is being used in some hatcheries is to allow fish to choose mates in specially designed spawning channels (Fig. 8.1), and it is believed better chromosome combinations may occur, especially after it has been demonstrated that fish mate according to their MH haplotypes (Landry et al. 2001; Eizaguirre et al. 2009). Because MH genes are related with disease resistance, it is interesting to see how the offspring of fish that are allowed mate choice will perform in challenge situations when compared to fish mated randomly, as is the common practice.

THE FUTURE OF BIOTECHNOLOGY AND IMMUNOLOGY IN AQUACULTURE

The world population is increasing, placing more pressure on food production. Traditional fisheries are struggling to provide the higher demands in the last decades due to improper management of natural resources, which has led to the collapse of many fisheries. Aquaculture is an alternative protein source to

Figure 8.1. Spawning channels used for chinook salmon. These are approximately 4 m wide, 15 m long, and, when being utilized, are filled to a depth of between 0.5 m to 1 m, with continuous flow to mimic a stream environment. These channels have been drained to show the pebbles lining the bottom, which mimic the preferred spawning habitat of wild chinook in streams.

compensate for the lack of natural fisheries and is growing rapidly. Farmed fish are expensive to feed and maintain, and many of them are susceptible to an array of diseases, which makes fish immunology a key area of study in order to assist aquaculturists to achieve success. As noted above, however, for many species, immune genes are virtually unknown and thus large-scale projects focused on gene discovery are needed. Genome projects are an effective route to take for this purpose. For example, the Cod Genomics and Broodstock Development project (http://codgene.ca/) sponsored by Genome Canada is mapping the genome of Atlantic cod with a focus on identifying genes related to growth rate and resistance to stress and disease. However, the identification of genes responsible for physiological and immunological traits that improve the performance of the fish constitutes is just the start of the process. The challenge once genes have been identified is first understanding their function, particularly if it is similar or differs from known mammalian counterparts, and second to move from that knowledge to practical and realistic methods for monitoring the health status of fish.

Once those genes that participate in immune responses have been identified, they can be included as target genes in future monitoring programs to evaluate their use in the early detection of hatchery outbreaks.

A good starting point, given the knowledge and techniques available today, would be to establish monitoring laboratories funded by both government and private sectors, with professional support from universities in an integrated effort to understand the problems and emergence of fish diseases. Of course, as the Red Queen hypothesis (Lively et al. 2004) says, pathogens are constantly evolving, so it is necessary to constantly monitor for the potential appearance of new strains of current diseases and novel pathogens. This is the real reason why these monitoring laboratories will need the constant support of fish immunologists and pathologists from universities and government.

SUMMARY

Biotechnology has a very promising future in the field of recombinant vaccines and can be used for monitoring fish health. Although a lot of progress has been made in aquaculture biotechnology, a deeper understanding of fish immunology is still necessary in order to create more sensitive and accurate analytical techniques that will provide a comprehensive and accurate measure of fish health status. More research into fish immunology is required in the years to come if growth in aquaculture is to meet demand in the coming decades.

REFERENCES

Afonso A, Ellis AE, and Silva MT. 1997. The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immun. 7: 335–348.

Alexander JB and Ingram GA. 1992. Non-cellular non-specific mechanisms in fish. Annu Rev Fish Dis. 2: 249–279.

Allendorf FW and Thorgaard GH. 1984. Tetraploidy and the evolution of Salmonid fishes. In: Evolutionary Genetics of Fishes (ed. Turner BJ). Plenum Press, New York. pp 1–53.

Anderson DP. 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annu Rev Fish Dis. 2: 281–307.

Arakawa H, Hauschild J, and Buerstedde J-M. 2002. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. Science. 295: 1301–1306.

Arkoosh MR and Kaattari SL. 1991. Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. Dev Comp Immunol. 15: 279–293.

Avrameas S and Ternynck T. 1995. Natural autoantibodies: the other side of the immune system. Res Immunol. 146: 235–248.

Barreto VM, Pan-Hammarstrom Q, Zhao Y, Hammarstrom L, Misulovin Z, and Nussenzweig MC. 2005. AID from bony fish catalyzes class switch recombination. J Exp Med. 202: 733–738.

Bagni M, Archetti L, Amadori M, and Marino G. 2000. Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J Vet Med B. 47: 745–751.

Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, and Schall TJ. 1997. A new class of membrane-bound chemokine with a CX3C motif. Nature. 385: 640–644.

Bernatchez L and Landry C. 2003. MHC studies in non-model vertebrates: what have we learned about natural selection in 15 years? J Evol Biol. 16: 363–377.

Bhon JA and Be Miller JN. 1995. (1–3)-β-D-glucan as biological response modifiers: a review of structure–function activity relationships. Carbohydr Polym. 28: 3–14.

Boes M. 2000. Role of natural and immune IgM antibodies in immune responses. Mol Immunol. 37: 1141–1149.

Boshra H, Li J, and Sunyer JO. 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immun. 20: 239–262.

Boshra H, Wang T, Hove-Madsen L, Hansen J, Li J, Matlapudi A, Secombes CJ, Tort L, and Sunyer JO. 2005. Characterization of a C3a receptor in rainbow trout and *Xenopus*: the first identification of C3a receptors in nonmammalian species. J Immunol. 175: 2427–2437.

Bricknell I and Dalmo RA. 2005. The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immun. 19: 457–472.

Burrells C, Williams PD, and Forno PF. 2001. Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids. Aquaculture. 199: 159–169.

Cannon JP, Haire RN, Rast JP, and Litman GW. 2004. The phylogenetic origins of the antigen-binding receptors and somatic diversification mechanisms. Immunol Rev. 200: 12–22.

Carroll RL. 1988. Vertebrate paleontology and evolution. Freeman, New York.

Castillo J, Teles M, Mackenzie S, and Tort L. 2009. Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (*Sparus aurata*). Fish Shellfish Immun. 27: 493–499.

Cossarini-Dunier M. 1986. Secondary response of rainbow trout (*Salmo gairdneri* Richardson) to DNP-haemocyanin and *Yersinia ruckeri*. Aquaculture. 52: 81–86.

Cuesta A, Rodriguez A, Esteban MA, and Meseguer J. 2005. In vivo effects of propolis, a honeybee product, on gilthead seabream innate immune responses. Fish Shellfish Immun. 18: 71–80.

Daeron M, Jaeger S, Du Pasquier L, and Vivier E. 2008. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev. 224: 11–43.

Diatchenko L, Lau YC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, and Siebert PD. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA. 93: 6025–6030.

Díaz-Rosales P, Bird S, Wang TH, Fujiki K, Davidson WS, Zou J, and Secombes CJ. 2009. Rainbow trout interleukin-2: cloning, expression and bioactivity analysis. Fish Shellfish Immun. 27: 414–422.

Dixon B and Stet RJM. 2001. The relationship between major histocompatibility receptors and innate immunity in teleost fish. Dev Comp Immunol. 25: 683–699.

Dixon B, Shum BPS, Adams EJ, Magor KE, Hedrick RP, Muir DG, and Parham P. 1998. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol Rev. 166: 345–352.

Dixon B, van Erp SHM, Rodrigues PNS, Egberts E, and Stet RJM. 1995. Fish major histocompatibility complex genes: an expansion. Dev Comp Immunol. 19: 109–133.

Du Pasquier L. 1982. Antibody diversity in lower vertebrates–why is it so restricted? Nature. 296: 311–313.

Eizaguirre C, Yeates SE, Lenz TL, Kalbe M, and Milinski M. 2009. MHC-based mate choice combines good genes and maintenance of MHC polymorphism. Mol Ecol. 18: 3316–3329.

Ellis AE. 1999. Immunity to bacteria in fish. Fish Shellfish Immmun. 9: 291–308.

Ellis AE. 2001. Innate host defence mechanism of fish against viruses and bacteria. Dev Comp Immunol. 25: 827–839.

Engstad RE and Robertsen B. 1995. Effect of structurally diffrent β-glucans on immune responses in Atlantic salmon (*Salmo salar* L.). J Mar Biotech. 3: 203–207.

Fast MD, Sims DE, Burka JF, Mustafa A, and Ross NW. 2002. Skin morphology and humoral nonspecific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp Biochem Physiol. A 132: 645–657.

Flajnik MF. 2002. Comparative analysis of immunoglobulin genes: surprises and portents. Nat Rev Immunol. 2: 688–698.

Flajnik MF. 2004. Another manifestation of GOD. Nature. 430: 157–158.

Fujiki K, Shin D-H, Nakao M, and Yano T. 1999. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1 and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics. 49: 909–914.

Fujiki K, Shin D-H, Nakao M, and Yano T. 2000a. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity

immunoglobulin E Fc receptor γ subunit and serum amyloid A. Fish Shellfish Immun. 10: 229–242.

Fujiki K, Shin D-H, Nakao M, and Yano T. 2000b. Molecular cloning of carp (*Cyprinus carpio*) leucocyte cell-derived chemotaxin 2, glia maturation factor β, CD45 and lysozyme C by use of suppression subtractive hybridization. Fish Shellfish Immun. 10: 643–650.

Gioacchini G, Smith P, and Carnevali O. 2008. Effects of Ergosan on the expression of cytokine genes in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to enteric red mouth vaccine. Vet Immunol Immunop. 123: 215–222.

Grimholt U, Larsen S, Nordmo R, Midtlyng P, Kjoeglum S, Storset A, Saebo S, and Stet RJM. 2003. MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. Immunogenetics. 55: 210–219.

Gudmundsdottir S, Magnadottir B, Bjornsdottir B, Arnadottir H, and Gudmundsdottir BK. 2009. Specific and natural antibody response of cod juveniles vaccinated against *Vibrio anguillarum*. Fish Shellfish Immun. 26: 619–624.

Guselle NJ, Speare DJ, Markham RJF, and Patelakis S. 2010. Efficacy of intraperitoneally and orally administered ProVale, a yeast β-(1,3)/(1,6)-D-glucan product, in inhibiting Xenoma formation by the microsporidian *Loma salmonae* on Rainbow trout gills. N Am J Aquacult. 72: 65–72.

Hahn HP and von Specht B-U. 2003. Secretory delivery of recombinant proteins in attenuated Salmonella strains: potential and limitations of type I protein transporters. FEMS Immunol Med Mic. 37: 87–98.

Hansen JD, Landis Ed, and Phillips RB. 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc Nat Acad Sci. 102: 6919–6924.

Hine PM. 1992. The granulocytes of fish. Fish Shellfish Immun. 2: 79–98.

Hordvik I. 1998. The impact of ancestral tetraploidy on antibody heterogeneity in salmonid fishes. Immunol Rev. 166: 153–157.

Hornick C and Karush F. 1972. Antibody affinity III. The role of multivalence. Immunochemistry. 9: 325–340.

Hsu E. 1998. Mutation, selection, and memory in B lymphocytes of exothermic vertebrates. Immunol Rev. 162: 25–36.

Hughes AL and Nei M. 1988. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature. 335: 167–170.

Iwanowicz LR, Blazer V, McCormick SD, VanVeld PA, and Ottinger, CA. 2009. Aroclor 1248 exposure leads to immunomodulation, decreased disease resistance and endocrine disruption in the brown bullhead, *Ameiurus nebulosus*. Aquatic Toxicology. 93: 70–82.

Jansson E, Lindberg L, Säker E, and Aspán A. 2008. Diagnosis of bacterial kidney disease by detection of *Renibacterium salmoninarum* by real-time PCR. J Fish Dis. 31: 755–763.

Jeney G and Jeney Z. 2002. Application of immunostimulants for modulation of the non-specific defense mechanisms in sturgeon hybrid: *Acipenser ruthenus* X *A. baerii*. J Appl Ichthyol. 18: 416–419.

Jones JM and Simkus C. 2009. The roles of the RAG1 and RAG2 "non-core" regions in V(D)J recombination and lymphocyte development. Arch Immunol Ther Exp. 57: 105–116.

Kaattari S, Evans D, and Klemer J. 1998. Varied redox forms of teleost IgM: an alternative to isotypic diversity? Immunol Rev. 166: 133–142.

Kaattari SL, Zhang Hl, Khor IW, Kaattari IM, and Shapiro DA. 2002. Affinity maturation in trout: clonal dominance of high affinity antibodies late in the immune response. Dev Comp Immunol. 26: 191–200.

Kubitza F, Lovshin LL, and Lovell RT. 1997. Identification of feed enhancers for juvenile largemouth bass *Micropterus salmoides*. Aquaculture. 148: 191–200.

Landry C, Garant D, Duchesne P, and Bernatchez L. 2001. "Good genes as heterozygosity": the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). Proc Biol Sci. 268: 1279–1285.

Langefors Å, Lohm J, Grahn M, Andersen Ø, and von Schantz T. 2001. Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. Proc R Soc Lond. B 268: 479–485.

Lei L, Fujiki K, Dixon B, and Sundick RS. 2002. Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing AU-rich elements. Cytokine. 17: 71–81.

Leong JC, Trobridge GD, Kim CHY, Johnson M, and Simon B. 1998. Interferon-inducible Mx proteins in fish. Immunol Rev. 166: 349–363.

Leung MYK, Liu C, Koon JCM, and Fung KP. 2006. Polysaccharide biological response modifiers. Immunol Lett. 105: 101–114.

Li J, Barreda DR, Zhang Y-A, Boshra H, Gelman AE, LaPatra S, Tort L, and Sunyer JO. 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat Immunol. 7: 1116–1124.

Lively CM, Dybdahl MF, Jokela J, Osnas EE, and Delph LF. 2004. Host sex and local adaptation by parasites in a snail-trematode interaction. Am Nat. 164(Suppl 5): S6–S18.

Lohm J, Grahn M, Langefors A, Andersen Ø, Storset A, and von Schantz T. 2002. Experimental evidence for major histocompatibility complex-allele-specific resistance to a bacterial infection. Proc R Soc Lond. B 269: 2029–2033.

Low C, Wadsworth S, Burrells C, and Secombes CJ. 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture. 221: 23–40.

Lund V, Børdalb S, Kjellsenb O, Mikkelsena H, and Schrøder MB. 2006. Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Aeromonas salmonicida* and *Vibrio anguillarum*. Dev Comp Immunol. 30: 1145–1155.

Malacek K, Brandman J, Brodsky JE, Ohta Y, Flajnik M, and Hsu E. 2005. Somatic hypermutation and junctional diversification at Ig heavy chain loci in the nurse shark. J Immunol. 175: 8105–8115.

Martin SW and Goodnow CC. 2002. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. Nat Immnunol. 3: 182–188.

Matejusová I, Felix B, Sorsa-Leslie T, Gilbey J, Noble LR, Jones CS, and Cunningham CO. 2006. Gene expression profiles of some immune relevant genes from skin of susceptible and responding Atlantic salmon (*Salmo salar* L.) infected with *Gyrodactylus salaris* (Monogenea) revealed by suppressive subtractive hybridization. Int J Parasitol. 36: 1175–1183.

Medzhitov R and Janeway CA Jr. 1997. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol. 9: 4–9.

Medzhitov R and Janeway CA Jr. 2000. How does the immune system distinguish self from nonself? Semin Immunol. 12: 185–188.

Medzhitov R and Janeway CA Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. Science. 296: 298–300.

Miller N, Wilson M, Bengtén E, Stuge T, Warr G, and Clem W. 1998. Functional and molecular characterization of teleosts leukocytes. Immunol Rev. 166: 187–197.

Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, and Honjo T. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 102: 553–563.

Nakanishi T, Kiryu I, and Ototake M. 2002. Development of a new vaccine delivery method for fish: percutaneous administration by immersion with application of a multiple puncture instrument. Vaccine. 20: 3764–3769.

Nath S, Fujiki K, Kales SC, and Dixon B. 2006. Major histocompatibility class II genes in rainbow trout (*Oncorhynchus mykiss*) exhibit temperature-dependent downregulation. Immunogenetics. 58: 443–453.

Nelson JS. 1994. Fishes of the World. 3rd ed. Wiley, New York.

Nomiyama H, Hieshima K, Osada N, Kato-Unoki Y, Otsuka-Ono K, Takegawa S, Izawa T, Yoshizawa A, Kikuchi Y, Tanase S, Miura R, Kusuda J, Nakao M, and Yoshie O. 2008. Extensive expansion and diversification of the chemokine gene family in zebrafish: identification of a novel chemokine subfamily CX. BMC Genomics 9: 222–240.

Powell DB, Palm RC Jr, MacKenzie AP, and Winton JR. 2009. Extremophile extracts and enhancement techniques show promise for the development of a live vaccine against *Flavobacterium columnare*. Cryobiology. 59: 158–163.

Rebl A, Goldammer T, and Seyfert H-M. 2010. Toll-like receptor signaling in bony fish. Vet Immunol Immunopath. 134(3–4): 139–150.

Robertsen B. 1999. Modulation of the non-specific defence of fish by structurally conserved microbial polymers. Fish Shellfish Immun. 9: 269–290.

Robertsen B, Rørstad G, Engstad R, and Raa J. 1990. Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J Fish Dis. 13: 391–400.

Rodrigues PNS, Vázquez-Dorado S, Neves JV, and Wilson JM. 2006. Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). Dev Comp Immunol. 30: 1156–1167.

Rodriguez A, Cuesta A, Esteban MA, and Meseguer J. 2004. The effect of dietary administration of the fungus *Mucor circinelloides* on non-specific immune responses of gilthead seabream. Fish Shellfish Immun. 16: 241–249.

Sakai DK. 1992. Repertoire of complement in immunological defense mechanisms of fish. Annu Rev Fish Dis. 2: 223–247.

Savan R and Sakai M. 2002. Analysis of expressed sequence tags (EST) obtained from common carp, *Cyprinus carpio* L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concanavalin-A. Comp Biochem Physiol B Biochem Mol Biol. 131: 71–82.

Secombes CJ. 1994. Enhancement of fish phagocyte activity. Fish Shellfish Immun. 4: 421–436.

Secombes CJ. 2008. Will advances in fish immunology change vaccination strategies? Fish Shellfish Immun. 25: 409–416.

Secombes CJ. 2009. Evolution and immunoregulation in vertebrates—perspectives from a fish cytokine biologist. Plenary Lecture. 11th Congress of the International Society for Comparative and Developmental Immunology, Prague, Czech Republic, July 3, 2009.

Shand R and Dixon B. 2001. Teleost major histocompatibility genes: diverse but not complex. Mod Asp Immunobiol. 2: 66–72.

Shephard KL. 1994. Functions for fish mucus. Rev Fish Biol Fish. 4: 401–429.

Shao M, Ma Y, Liu Q, and Zhang Y. 2005. Secretory expression of recombinant proteins in an attenuated *Vibrio anguillarum* strain for potential use in vaccines. J Fish Dis. 28: 723–728.

Sher A and Coffman RL. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu Rev Immunol. 10: 385–409.

Sinyakov MS, Dror M, Zhevelev HM, Margel S, and Avtalion RR. 2002. Natural antibodies and their significance in active immunization and protection against a defined pathogen in fish. Vaccine. 20: 3668–3674.

Skjermo J and Bergh Ø. 2004. High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using *Artemia* for delivery, increases resistance against vibriosis. Aquaculture. 238: 107–113.

Solem ST and Stenvik J. 2006. Antibody repertoire development in teleosts—a review with emphasis on salmonids and *Gadus morhua* L. Dev Comp Immunol. 30: 57–76.

Stavnezer J and Amemiya CT. 2004. Evolution of isotype switching. Sem Immunol. 16: 257–275.

Stet RJM, Dixon B, van Erp SHM, van Lierop M-JC, Rodrigues PNS, and Egberts E. 1996. Inference of structure and function of fish major histocompatibility complex (MHC) molecules from expressed genes. Fish Shellfish Immun. 6: 305–318.

Stet RJM, Kruiswijk CP, and Dixon B. 2003. Major histocompatibility lineages and immune gene function in teleost fishes: the road not taken. Cr Rev Immunol. 23: 441–471.

Sunyer JO and Lambris JD. 1998. Evolution and diversity of the complement system of poikilothermic vertebrates. Immunol Rev. 166: 39–57.

Sunyer JO, Zarkadis IK, and Lambris JD. 1998. Complement diversity: a mechanism for generating immune diversity? Immunol Today. 19: 519–523.

Tangye SG and Tarlinton DM. 2009. Memory B cells: effectors of long-lived immune responses. Eur J Immunol. 39: 2065–2075.

True K, Purcell MK, and Foott JS. 2008. Development and validation of a quantitative PCR to detect *Parvicapsula minibicornis* and comparison to histologically ranked infection of juvenile chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), from the Klamath River, USA. J Fish Dis. 32: 183–192.

Tsoi SC, Ewart KV, Penny S, Melville K, Liebscher RS, Brown LL, and Douglas SE. 2004. Identification of immune-relevant genes from Atlantic salmon using suppression subtractive hybridization. Mar Biotechnol. 6: 199–214.

Verlhac V, Gabaudan J, Obach A, Schüep W, and Hole R. 1996. Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 143: 123–133.

Verlhac V, Obach A, Gabaudan J, Schüep W, and Hole R. 1998. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immun. 8: 409–424.

Villamil L, Figueras A, and Novoa B. 2003. Immunomodulatory effects of nisin in turbot (*Scophthalmus maximus* L.). Fish Shellfish Immun. 14: 157–169.

Voss EW and Sigel MM. 1972. Valence and temporal change in affinity of purified 7S and 18S nurse shark anti-2,4-dinitrophenil antibodies. J Immunol. 109: 665–673.

Wang C, Zhang X-H, Jia A, Chen J, and Austin B. 2008. Identification of immune-related genes from kidney and spleen of turbot, *Psetta maxima* (L.), by suppression subtractive hybridization following challenge with *Vibrio harveyi*. J Fish Dis. 31: 505–514.

Wang HR, Hu YH, Zhang WW, and Sun L. 2009. Construction of an attenuated *Pseudomonas fluorescens* strain and evaluation of its potential as a cross-protective vaccine. Vaccine. 27: 4047–4055.

Warr GW. 1995. The immunoglobulin genes of fish. Dev Comp Immunol. 19: 1–12.

Wilson MR and Warr GW. 1992. Fish immunoglobulins and the genes that encode them. Annu Rev Fish Dis. 2: 201–221.

Wilson M, Bengtén E, Miller NW, Clem LW, Du Pasquier L, and Warr GW. 1997. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proc Natl Acad Sci USA. 94: 4593–4597.

Yang F, Waldbieser GC, and Lobb CJ. 2006. The nucleotide targets of somatic mutation and the role of selection in immunoglobulin heavy chains of a teleost fish. J Immunol. 176: 1655–1667.

Young KM, Czyrny A, Russell S, Huber P, and Lumsden JS. 2009. Plasma ladderlectin concentration following sterile inflammation and *Aeromonas salmonicida* subsp. *salmonicida* infection. J Fish Dis. 32: 569–576.

Zapata A and Amemiya CT. 2000. Phylogeny of lower vertebrates and their immunological structures. Curr Top Microbiol Immunol. 248: 67–110.

Zarkadis IK, Mastellos D, and Lambris JD. 2001. Phylogenetic aspects of the complement system. Dev Comp Immunol. 25: 745–762.

Zuo X and Woo PTK. 1997. In vivo neutralization of *Cryptobia salmositica* metallo-protease by α2-macroglobulin in the blood of rainbow trout *Oncorhynchus mykiss* and in brook charr *Salvelinus fontinalis*. Dis Aquat Org. 29: 67–72.

Part 4
Viral Pathogens and Diseases

Chapter 9

Structural Biology and Functional Genomics of the Shrimp White Spot Syndrome Virus and Singapore Grouper Iridovirus

Jinlu Wu, Zhengjun Li, and Choy L. Hew

INTRODUCTION

The aquaculture industry is threatened by a variety of viral diseases. The white spot syndrome virus (WSSV) and Singapore grouper iridovirus (SGIV) are two major pathogens that we have been studying. Both WSSV and SGIV are large dsDNA viruses whose genomes have been sequenced and analyzed. However, most of the predicted encoded viral proteins have not been identified and their functions remain elusive (Yang et al. 2001; Song et al. 2004). Recently, the rapid development of proteomics technologies, including protein separation techniques and mass spectrometry, has facilitated the identification, quantification, and characterization of proteins (Khalsa-Moyers and McDonald 2006). Similarly, technological advances in X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are changing the way in which protein structures can be determined and protein–ligand interactions characterized (Bonvin 2006). Structural analysis can facilitate the study of novel protein function in a number of different ways, such as inferring functional details by comparison of the function of other proteins adopting similar fold or key protein-binding sites (Aravind et al. 2006). X-ray crystallography and NMR spectroscopy are two common and complementary platforms for structural determination (Pusey et al. 2005). Here, we present how these approaches were effectively integrated to work on virus protein identification, viral protein structure determination, and host–virus interactions using WSSV and SGIV as models.

Shrimp White Spot Syndrome Virus

Shrimp aquaculture has become an important industry worldwide during the last few decades. However, intensive cultivation, inadequate sanitation of the shrimp industry, and worldwide trade of live stock have unfortunately promoted the spread of viral pathogens. Out of the more than 20 viral agents reported, WSSV is the most serious pathogen and has caused mass mortalities in cultured shrimp

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

worldwide. The initial and major target of this virus is the shrimp epithelia, including subcuticular, stomach, and gill epithelia. WSSV-infected epithelial cells show hypertrophied nuclei containing massive amounts of viruses (Lightner 1996). Clinical signs of the disease include a red color covering the entire body and appendages along with small subcutaneous white spots. WSSV has a broad host range, infecting several crustacean species, including shrimp, crabs, and crayfish (Wang et al. 1998). WSSV particles are nonoccluded and bacilliform in shape with double envelopes belonging to the family *Nimaviridae* (genus *Whispovirus*). Its genome consists of a 305,107 base pair (bp), double-stranded circular DNA that encodes 181 predicted open reading frames (ORFs) (Yang et al. 2001). Most of these ORFs are of unknown function as no homologs to known genes can be found in public databases.

Singapore Grouper Iridovirus

Iridoviruses are large dsDNA viruses that infect many invertebrates and poikilothermic vertebrates (Williams et al. 2005). Some viral strains have been recognized as key pathogens in wild and farmed fish, reptile, and amphibian species. Since the first discovery of iridovirus in 1954, more than 100 of them have been isolated and classified into five genera within the family *Iridoviridae*. The virus replicates in cell cytoplasm. Matured viral particles show icosahedral shape and can be separated into three layers: the capsid, lipid layer, and a DNA–protein complex (Yan et al. 2000). In 1994, a novel viral disease called sleepy grouper disease resulted in significant economic losses in Singapore marine net cage farms and an iridovirus, designated SGIV, was identified as the causative pathogen in 1998. On the basis of the virus characteristics, such as the sources of host organisms, genetic properties, and morphology, SGIV was assigned into the genus *Ranavirus*. The entire SGIV genome consists of a dsDNA of 140,131 bp with 162 predicted ORFs (Song et al. 2004). To date, 12 genomes of iridoviruses have been completely sequenced whose sizes vary between 105 and 212 kbp. Despite the abundance of genomic information, studies on iridoviruses are largely limited to ORF prediction and cross-species genome comparisons. The authenticity of an ORF as a functional protein would need to be verified experimentally. So far, very little work has been carried out on the biological functions of iridovirus genes and gene products, except for a few highly conserved genes such as the major capsid protein and ATPase (Eaton et al. 2007).

PROTEOMICS STUDIES ON WSSV AND SGIV

General Description of Proteomics Technology

Proteomics is a powerful tool for the rapid and comprehensive evaluation of protein profiles in complex protein samples, the analysis of protein interactions, and the characterization of posttranslationally modified proteins. It not only confirms the presence of the protein but also provides a direct measure of the quantity present. The identification of the proteins and their distributions in cells contribute greatly to the understanding of cellular function. The completion of multiple genome projects has stimulated the rapid development of the proteomics technologies. In gel-based proteomics, proteins are separated by one- or two-dimensional gel electrophoresis (1D or 2D GE) before proteolytic digestion. Gel-free proteomics uses liquid chromatography (LC) to separate peptides after proteolytic protein cleavage in solution. The bottom-up and the top-down approach are two principal proteomics approaches (Guerrera and Kleiner 2005). The bottom-up approach is a common method to identify proteins. Protein mixtures are enzymatically digested into complex peptide mixtures, which are then fractionated by multidimensional high-pressure LC (LC/LC) before they are subjected to tandem mass spectrometry (MS/MS). This technique is also known as shotgun proteomics. It enables the direct analysis of complex protein mixtures to rapidly generate a global profile of the protein complement within the mixtures. In the top-down approach, protein mixtures are fractionated and intact proteins are displayed before mass spectrometric identification and characterization. This approach requires more complex instrumentation and methodology than the more widely used bottom-up approach, but the top-down data are more specific.

Protein quantification is necessary for understanding of transcriptional, translational, and posttranslational effects that affect protein production and function. The ability to quantify proteins of interest is another key feature of proteomic studies. Recent developments in stable isotope labeling techniques and chemical tagging allow the study of mass spectrometer-based differential displays and the quantification of protein expression. Stable isotopes can be labeled by either biosynthetic or chemical methods. A cleavable isotope-coded affinity tag (ICAT) approach is one of the chemical labeling techniques. It involves cysteine-specific tagging of intact proteins prior to proteolytic digestion and enables the quantitative pairwise comparison of protein expression levels under different conditions (Booy et al. 2005). However, ICAT is not applicable to cysteine-free proteins or proteins in which cysteines are not accessible by the ICAT reagent. Isobaric tags for relative and absolute quantification (iTRAQ) is a newly developed and promising LC-based quantitative proteomics approach. The iTRAQ reagents are isobaric reagents that are amine specific and can be multiplexed to allow up to four samples to be quantified simultaneously (Ross et al. 2004). This technique significantly eliminates experimental variations between different mass spectrometer runs that could be introduced upon independent sample analysis and reduces the time of analysis. In contrast to ICAT, stable isotopes introduced by iTRAQ reagents occur at the level of proteolytic peptides. The amine-specific peptide labeling can be carried out without loss of information from samples involving post-translational modifications. This technique can be applied to temporal analysis of differential expression of proteins in perturbed systems induced by pathogens or drugs, discovery and elucidation of disease markers, and protein–protein interactions in multiprotein complexes. The qualitative and quantitative analysis of global proteome samples has had a big impact on the understanding of protein function.

Viral Proteomics of WSSV and SGIV

The complexity of viral pathogenesis involves many factors, including viral structural proteins and non-structural proteins. Understanding viral pathogenesis is the key to developing treatments for viral diseases. The relatively small size of the viral genome makes it an ideal model system for structural and functional studies using proteomic techniques. Many different proteomic approaches have been utilized to study the virus components and to globally monitor the impact of viral infection on the proteome of the host cell in order to dissect the mechanism of viral pathogenesis. So far, there are two major areas of viral proteomics. The first one is virion proteomics: the characterization of viral protein components in order to understand virus assembly. The second is the analysis of changes in the host cell proteome and the interaction between viruses and their host during viral infection.

Characterization of Viral Protein Components

Because of the relatively low complexity of proteins in the virion, viral protein mixtures can be separated by 1D GE and further identified by peptide mass fingerprinting or MS/MS. Gel-based proteomic approaches have been applied in WSSV proteomic studies (Huang et al. 2002; Tsai et al. 2004; Zhang et al. 2004; Xie et al. 2006). The first proteomics study of WSSV successfully identified 18 structural proteins on 1D GE (Huang et al. 2002). In a subsequent study, 33 WSSV structural proteins resolved by 1D GE were further identified using liquid chromatography-nano-electrospray ionization tandem mass spectrometry (LC-nanoESI-MS/MS) (Tsai et al. 2004). Altogether, these four gel-based proteomics studies on WSSV have identified 39 structural proteins. Similarly, the iridoviral proteome has also been partially characterized using matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry, identifying 26 structural proteins from SGIV following 1D GE separation (Song et al. 2004).

Recently, both the WSSV and SGIV proteomes were further investigated by shotgun proteomics using offline coupling of the LC system with MALDI TOF/TOF MS/MS, and a more comprehensive identification of the viral proteins was achieved (Song et al. 2006; Li et al. 2007). Shotgun proteomics of

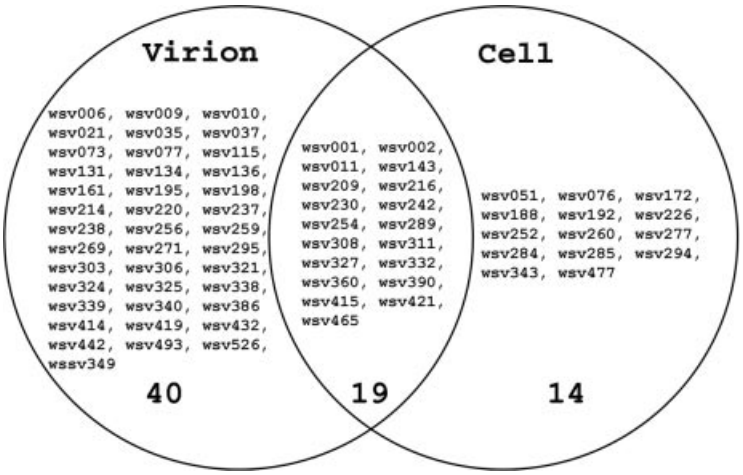


Figure 9.1. List of identified structural and nonstructural WSSV proteins. Totally, 73 proteins are identified. Among them, 59 of them can be found in the virion, 33 of them in the WSSV-infected cell, and 19 of them in both the virion and the WSSV-infected cell.

WSSV identified 45 viral proteins, 13 of which were reported for the first time. Hence, WSSV contains at least 59 structural proteins. Novel viral proteins identified in infected shrimp epithelial cells were also identified (Wu et al. 2007) (Fig. 9.1). Some of the viral proteins identified are nonstructural, as validated by Western blots.

An additional 25 SGIV viral proteins have been detected by the shotgun approach, and 13 were found to have acetylated N termini. So far, a total of 51 SGIV structural proteins have been detected by mass spectrometry (Fig. 9.2). In addition, iTRAQ analysis of SGIV-infected grouper embryonic cells identified 11 additional viral proteins for the first time (Chen et al. 2008; Fig. 9.2).

All of this information enriches our knowledge of viral structural proteins. Moreover, these studies suggest that both the gel-based approach and the LC-based shotgun approach are equally effective and complementary to each other.

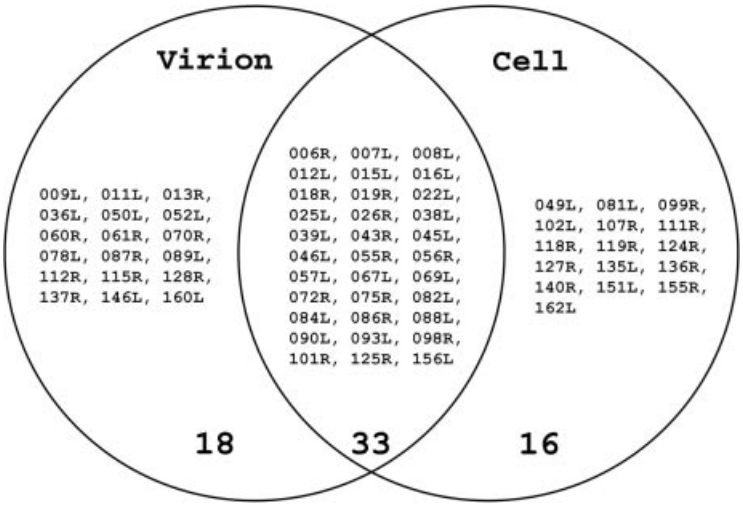


Figure 9.2. List of identified structural and nonstructural SGIV proteins. Totally, 67 proteins are identified. Among them, 51 of them can be found in the virion, 49 of them in the SGIV-infected cell, and 33 of them in both the virion and the SGIV-infected cell.

iTRAQ for Viral Protein Localization in WSSV

Since envelope and nucleocapsid viral proteins have their own distinct functions, the ability to discriminate them and thus to determine their location will help to elucidate their functions in viral pathogenesis. Immunogold electron microscopy and Western blot analysis are two common methods for determining the location of different viral proteins. However, both methods require the production of specific antibodies, which would require the proteins to be purified.

The iTRAQ approach has been used as a method to study the differential expression of proteins in perturbed systems. It has also been successfully applied to the study of enriched organelle proteins and to conduct protein correlation profiling (Chen et al. 2006; Foster et al. 2006). The identification of proteins within enriched subcellular compartments by mass spectrometry has been established as a rapid method of determining their location and function within cell organelles. To expand on the use of this application, we utilized the iTRAQ approach to identify and localize the WSSV envelope and nucleocapsid proteins. The localization of proteins within the virus has been hindered by difficulties in preparing pure envelope and nucleocapsid fractions (Xie et al. 2006).

We used iTRAQ reporter ions to quantify the enriched envelope proteins in the detergent solubilized fraction and the nucleocapsid proteins in the pellet fraction. The structural identities of these proteins were determined using MS/MS. Two distinct iTRAQ reporter ion spectra were obtained, one for the envelope proteins and the other for the nucleocapsid proteins (Li et al. 2007). This established that the iTRAQ methodology can discriminate between different groups of viral proteins quantitatively and hence determine their location. On the basis of iTRAQ ratios obtained from this study, 23 envelope proteins and 6 nucleocapsid proteins were identified (Table 9.1). The results also validated the presence of 15 structural proteins whose location within the virion was already known. Thus, iTRAQ is an effective approach for high-throughput viral protein localization determination. The significance of this high-throughput approach is that it does not rely on viral protein purification. Instead, it enables one to determine directly which proteins are components of the viral envelope and which are located in the nucleocapsoid. This approach can also be applied to study other enveloped viruses.

Identification of Differentially Expressed Host Proteins

ICAT Identification of Differentially Expressed Shrimp Proteins During WSSV Infection

Quantitative proteomics affords opportunities to concurrently identify and determine the expression levels of proteins in cells or tissues of different health status, e.g., healthy versus diseased. ICAT was used to study differentially expressed cellular proteins in WSSV-infected subcuticular epithelium of *Penaeus monodon* (Wu et al. 2007). Because of the fact that the shrimp genome data is incomplete, the number of cellular proteins identified was limited. Two upregulated and ten downregulated host proteins were detected from the ICAT-labeled shrimp epithelial lysate. During WSSV infection, hemocyanin was upregulated, which is consistent with its important role in antivirus defense. WSSV infection may induce the aggregation of hemocytes at the sites of infection (van de Braak et al. 2002). Upregulated clottable protein at the early infection stage may indicate an innate immune response of the host, while its downregulation at the late infection stage may explain why hemolymph from shrimp at this stage of infection does not coagulate. A more in-depth characterization of host–virus interactions would improve our understanding of the mechanism of pathogenicity and help define novel therapeutic targets.

iTRAQ Analysis of SGIV Infected Grouper Embryonic Cells

The iTRAQ technique coupled with LC–MS/MS was used to study the proteomic profiles of a brown-spotted grouper (*Epinephelus tauvina*) embryonic cell line before and after SGIV infection (Chen et al. 2008). Twelve host proteins were upregulated more than 1.5-fold and five host proteins were downregulated more than 1.5-fold upon viral infection. The increased expression of grouper mucin-associated surface protein, which is an important factor in the

Table 9.1. The Localization of Structural Proteins in WSSV.

No.	Name	Accession No.	Location in the Virion*	Localization Methods			Alternative Names
				MS†	WB‡	IEM	
1	wsv001	gi|17158634	E			IEM	WSSV-CLP
2	wsv002	gi|17158106	E/T	iTR	WB	IEM	VP24
3	wsv006	gi|17158110	E	iTR			
4	wsv009	gi|17158113	E	iTR			
5	wsv011	gi|17158115	E	iTR	WB		VP53A
6	wsv021	gi|17158125	E	iTR			
7	wsv035	gi|17158139	E		WB	IEM	VP110
8	wsv037	gi|17158141	NC	1D			VP160B
9	wsv077	gi|17158181	T		WB		VP36A
10	wsv115	gi|17158219	E	iTR			
11	wsv143	gi|17158247	NC	iTR			
12	wsv198	gi|17158302	E	iTR			
13	wsv209	gi|17158313	E	iTR	WB	IEM	VP187
14	wsv214	gi|17158318	NC		WB		VP15
15	wsv216	gi|17158320	E	iTR	WB	IEM	VP124
16	wsv220	gi|17158324	E		WB		VP674, VP73, VP76
17	wsv230	gi|17158334	E	iTR			
18	wsv237	gi|17158341	E		WB	IEM	VP292
19	wsv238	gi|17158342	E	iTR	WB		
20	wsv242	gi|17158346	E	iTR	WB		
21	wsv254	gi|17158357	E	iTR	WB	IEM	VP281, VP36B
22	wsv256	gi|17158359	E	iTR	WB		VP51B
23	wsv259	gi|17158362	E	iTR	WB		VP38A
24	wsv271	gi|17158374	NC	iTR			
25	wsv289	gi|17158391	NC	iTR, 1D			VP160A
26	wsv295	gi|17158397	E	iTR			
27	wsv306	gi|17158408	E/T	iTR	WB		VP39A
28	wsv308	gi|17158410	NC	iTR, 1D	WB	IEM	VP466, VP51C
29	wsv311	gi|17158413	E/T	iTR	WB	IEM	VP26
30	wsv321	gi|17158423	E	iTR			
31	wsv325	gi|17158427	E	iTR			
32	wsv327	gi|17158429	E	1D			
33	wsv339	gi|17158441	E		WB	IEM	VP39
34	wsv340	gi|17158442	E		WB	IEM	VP31
35	wsv360	gi|17158462	NC	iTR, 1D	WB	IEM	VP664
36	wsv386	gi|17158488	E			IEM	VP68
37	wsv414	gi|17158516	E		WB		VP19
38	wsv415	gi|17158517	NC	iTR, 1D	WB		VP60B
39	wsv421	gi|17158523	E	iTR	WB	IEM	VP28
40	wsv432	gi|17158534	E		WB	IEM	
41	wsv442	gi|17158543	E/T	iTR	WB		VP95
42	wsv493	gi|17158594	NC		WB		VP35
43	wssv349	gi|19481941	E	iTR			

*E, envelope; T, tegument; NC, nucleocapsid.

†iTR, detected by iTRAQ; 1D, detected by 1D-gel.

‡WB, Western blot.

host innate immune system, indicated that SGIV infection triggered innate immune responses. The upregulation of the cellular dihydrofolate synthase and the ATP synthase F1 alpha subunit implied that SGIV may hijack the host machinery for its replication. Furthermore, this study suggested that the virus may protect its own proteins by inhibiting the expression of the host proteases and ubiquitin. Alpha-2-macroglobulin isoform 1, which is involved in protease inhibition, was upregulated in SGIV-infected cells, while S27a protein, which forms a fusion protein with mono-ubiquitin, was downregulated. These observations are helping us to identify important cellular regulatory proteins and pathways during SGIV infection.

FUNCTIONAL ANALYSIS OF SGIV GENES USING MORPHOLINOS

Morpholino oligos are short chains of morpholino subunits comprising a nucleic acid base, a morpholine ring, and a nonionic phosphorodiamidate intersubunit linkage. Morpholinos block gene expression at the translation level and act via a steric block mechanism (RNAse H-independent), and with their high mRNA binding affinity and specificity, they yield reliable and predictable results (Summerton and Weller 1997). We designed antisense oligos using a web-based server (http://www.genetools.com/Oligo_Design) and used the Amaxa Nucleofection System to deliver the morpholinos.

SGIV gene ORF018R has been shown to be conserved in frog virus 3 (FV3), tiger frog virus, *Ambystoma tigrinum* virus (ATV), and grouper iridoviruses (Eaton et al. 2007). BLAST analyses have shown that the encoded protein contained a partial serine/threonine kinase catalytic domain, suggesting that this protein might be involved in phosphorylation processes. Moreover, this protein has been shown to occur at high abundance in mature SGIV virions, indicating an important role in virus infection (Song et al. 2004, 2006). The potential role of ORF018R was explored by knockdown of its expression via an antisense morpholino oligonucleotide (asMO) technique using grouper embryonic cells as the host.

The delivery of an asMO against ORF018R (asMO18) into grouper embryonic cells using Amaxa nucleofaction technology resulted in dramatically reduced expression of ORF018R that further led to (i) partial inhibition of expression of late viral genes and blocking of virus infection, (ii) distortion of viral DNA packaging and virion assembly, and (iii) enhanced serine phosphorylation (Wang et al. 2008a). Furthermore, five proteins with enhanced phosphorylation were identified using 2DE-MS analyses, of which three were viral proteins (ORF075R, ORF086R, and ORF049L (dUTPase)) and two were host proteins (natural killer enhancing factor and eukaryotic translation initiation factor 3 subunit 12) (Wang et al. 2008a). AsMOs were also successfully applied to inhibit the major capsid protein, the 18 kDa immediate-early protein, and a viral homolog of RNA polymerase II of FV3 (Sample et al. 2007).

STRUCTURAL CHARACTERIZATION OF NOVEL VIRAL PROTEINS

Target Selection and Expression

Although many structural genomic programs have used different criteria or priorities for the selection of the target proteins (Chandonia and Brenner 2005), the common theme is to restrict the selection to candidates of unknown structure, expected interest, and accessibility (e.g., yields, solubility, ease of labeling, etc.). In the research on WSSV and SGIV, we focused on the following: (i) envelope proteins important for virus assembly, structure, and initiation of viral infection and (ii) proteins of small molecular weight with unknown structure/function. These candidate proteins were expressed in a bacterial system using modified pET32a or pGEX6P-1 vector.

Structural Analysis of WSSV VP26 and VP28

Viral envelope proteins are important to initiate infection because they are believed to be the first molecules to interact with the host and, consequently, play critical roles in cell targeting as well as in triggering host defenses (Cheung et al. 2005). VP28 and VP26 are the two most abundant structural proteins observed in the WSSV envelope (Xie et al. 2006; Fig. 9.3). Preliminary studies showed that VP28 was crucial for virus entry and vaccine-induced protection (van Hulten et al. 2001; Namikoshia et al. 2004; Yi et al. 2004). Neither VP28 nor VP26 shares significant homology with

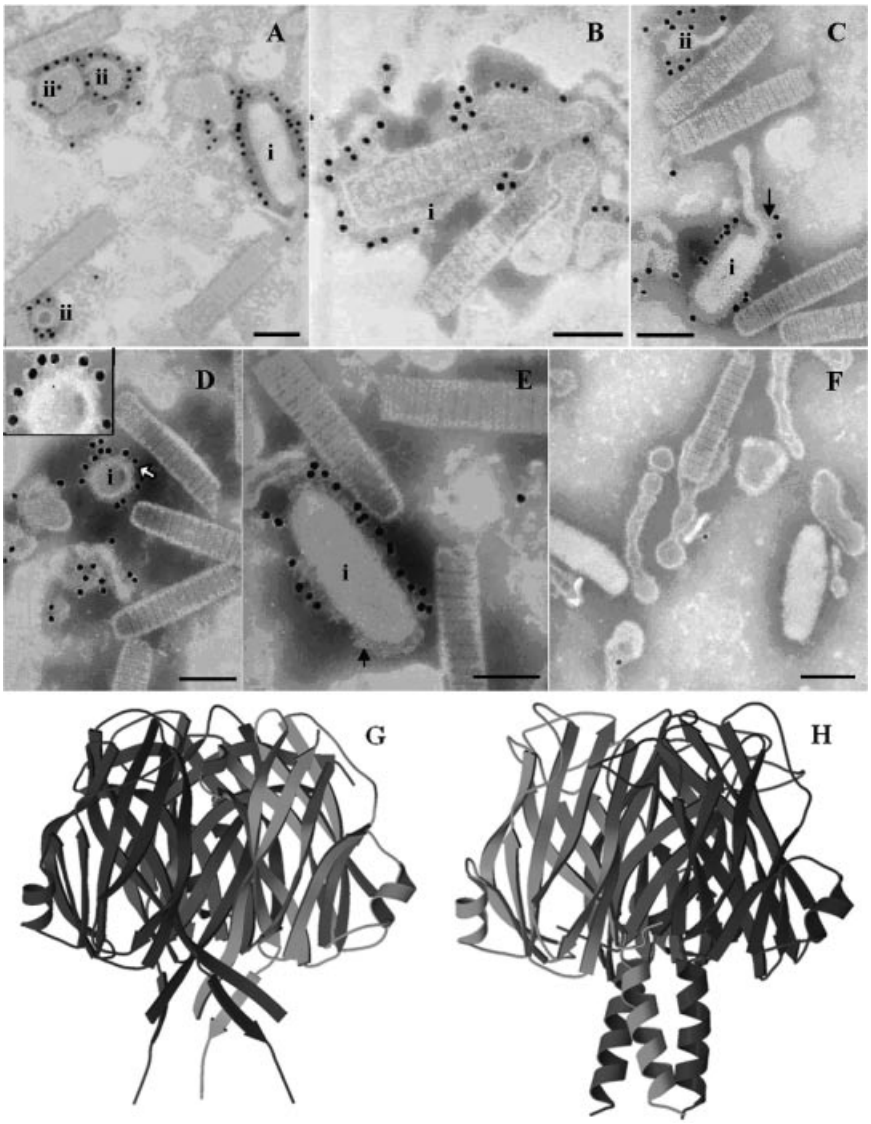


Figure 9.3. Localization and structure of WSSV envelope protein of VP26 and VP28. Purified WSSV virion was treated with 0.1% Tween 20 and then was immunogold labeled with VP26 and VP28 antibodies. Panels from **A** to **D** show VP26 antibody specifically binding to the outer surface with the gold particles localized on the outer surface of virions (**A**-i & **C**-i), semi-separated envelope (**B**-i), and completely separated envelope (envelope vacuoles, **A**-ii, **C**-ii, and **D**-i). Panel **E** shows that immunogold-labeled VP28 is also localized on the outer surface of the envelope. Surface-protruding structures (arrow) clearly appeared on some of the envelope and vesicles. The inset in panel **D** shows a large view of the surface protrusions. Panel **F** shows a very low level of nonspecific binding when a preimmune rabbit antibody or gold-conjugated secondary antibody was used. The image possibly also shows the process of vesicle formation. Panel **G** and **H** are the ribbon diagram of the VP26 and VP28, respectively. (Figures modified from Tang et al. 2007; Bar = 100 nm.) (For color detail, please see the color plate section.)

known structural proteins from other viruses. However, these two proteins share a sequence similarity of 41%. Further, WSSV envelope protein VP24 shares significant sequence similarities of 46% and 41% with VP28 and VP26, respectively, whereas WSSV envelope protein VP19 has no sequence similarity with either WSSV proteins or other known viral proteins.

Truncated constructs of VP26 and VP28 genes were amplified from the WSSV genome and inserted into vector pGEX6P-1 (Amersham). VP26 and VP28 proteins with glutathione S-transferase tags, expressed in *E. coli* BL21 (DE3), were purified by glutathione-Sepharose 4B (Amersham). Crystals of VP26 and VP28 were obtained using the hanging-drop method at 20°C.

Crystal structure of two major envelope proteins, VP26 and VP28, adopt similar β-barrel architectures with a protruding N-terminal region (Fig. 9.3) (Tang et al. 2007). The full-length VP26 consists of a nine-stranded β-barrel with mostly antiparallel β-strands and a two-turn-α-helix hanging outside the β-barrel structure. The pore of the β-barrel is highly hydrophobic in nature and the approximate dimensions of the β-barrel are 38.5Å (height) $\times$ 15.5Å (diameter). The full-length VP28 exhibits a single β-barrel and an α-helix protruding from the β-barrel architecture. The overall dimensions of the β-barrel are approximately 35 Å (height) $\times$ 15 Å (diameter). Similar to VP26, the β-barrel in VP28 is composed of 9 β-strands and is highly hydrophobic. Although the β-barrel architecture is one of the most commonly found structural features of viral proteins (Tang et al. 2007 and references therein), VP26 and VP28 are unlike the predominant eight-stranded β-barrels, but have a nine-stranded β-barrel, a feature not commonly observed in other viral proteins. This unique feature of WSSV proteins is consistent with the phylogenetic tree of the WSSV genes' DNA polymerase and shows that this virus is not related to any of the larger double-stranded DNA virus families. Immunoelectron microscopy investigation indicates that VP26 and VP28 are located on the virus envelope. The observed trimer shapes of the VP26 and VP28 crystal structures might be projected out for the interaction with the host cell for an effective transfer of viral infection (Tang et al. 2007).

Structural Analysis of WSSV VP9

VP9 is encoded by a predicted ORF of wsv230 (Yang et al. 2001; Liu et al. 2006). Real-time reverse transcription-PCR demonstrated that the transcription of VP9 started from the early to the late stage of WSSV infection with a high abundance. Western blotting revealed that the anti-VP9 antibody recognized a protein band of 9 kDa not from WSSV virion or noninfected tissues but only from the WSSV-infected stomach and gills. This band was analyzed by mass spectrometry, and its identity was confirmed as VP9 by N-terminal sequencing. Further, WSSV virions were separated into envelope and nucleocapsid fractions; however, anti-VP9 failed to recognize VP9 from both fractions. The observation that VP9 was detected only from the WSSV-infected tissues but not from purified virions suggests that VP9 is a nonstructural viral protein. That immunogold labeling using anti-VP9 antibody showing no gold labeling signal by transmission electron microscopy also backed our conclusion. Since VP9 is highly abundant at both transcript and protein levels and does not share homologs with reported nucleic acids and proteins, it is interesting to infer its potential function via three-dimensional (3D) structural analysis (Liu et al. 2006).

Recombinant VP9 was expressed in *E. coli* BL-21 cells and purified. Crystals of VP9 were obtained by using the hanging-drop vapor diffusion method. The 3D structures of VP9 were independently determined by X-ray crystallography and NMR spectroscopy (Liu et al. 2006). The root mean square deviation (rmsd) between X-ray and NMR models was $\sim$1.194 Å for 74 Cα atoms (Phe4 to Thr76), which indicated a good agreement between the two structures. It is worth mentioning that the NMR structure was determined in conditions free of metal ions, whereas the X-ray structure was determined with Cd2+ ions. The observed metal ion was located on the monomer interface of the dimer and also on the surface of the molecule in the crystal structure. This suggested that the metal ion was not essential for the folding of VP9. The crystal structure of VP9 was determined using the single wavelength anomalous dispersion method from synchrotron data and refined to a final R factor of 0.225 (Rfree = 0.275) at 2.35 Å resolution. The monomer of VP9 molecule adopts a mixed α/β fold with

overall dimensions of 32 Å × 25 Å × 20 Å. A total of six β-strands and two α-helices are found per molecule. It revealed a ferredoxin fold with divalent metal ion binding sites. Cadmium sulfate was found to be essential for crystallization. The Cd2+ ions were bound between the monomer interfaces of the homodimer. In the crystal, two dimers of one asymmetric unit are related by a twofold noncrystallographic symmetry. But in solution, VP9 exists in fast exchange equilibrium between monomer and symmetric dimer, which was demonstrated by gel filtration, dynamic light scattering, analytic ultracentrifuge, and NMR relaxation measurements.

Searches for structurally similar proteins within the protein data bank (PDB, http://www.rcsb.org/pdb/static.do?p=search/index.html) were performed with the program DALI (Holm and Sander 1995). Significant structural similarities were found with several nucleotide binding and metal transport/binding proteins. The highest structural similarity was observed between VP9 and bovine papillomavirus-1 E2 DNA-binding protein (PDB code 2BOP), yielding an rmsd of 3.0 Å for 63 Cα atoms, with 17% sequence identity. Therefore, we hypothesize that VP9 might be involved in the transcriptional regulation of WSSV, a function similar to that of the E2 protein during papillomavirus infection of the host cells. Recent studies suggest that VP9 might interfere with nucleosome assembly and prevent H2A.x from fulfilling its critical function of repairing DNA double strand breaks (Wang et al. 2008b).

SUMMARY

Recent advances in proteomic approaches have greatly facilitated the detection of virion components, changes in the cellular proteome upon viral infection, and elucidated protein interactions between virus and host. These findings provide valuable information regarding how a virus changes its host proteome to adapt to the host cell and escape from immune surveillance. On the other hand, structural biology has provided new insights into the functions of novel viral proteins. Information acquired using proteomic and structural biology approaches will lead to a more comprehensive picture of the mechanism of viral infection and could aid in the discovery of specific viral inhibitors that will lead to effective antiviral therapies.

ACKNOWLEDGMENTS

We thank our graduates Drs. Wenjun Song, Jing Chen, Xuhua Tang, Yang Liu, Liming Chen, Fan Wang, and Bich Ngoc Tran for contributing most of the data used in this review. Our research was supported by Research Grants R-154-000-223-112, R-154-000-255-112, and R-154-000-387-112 to Choy-Leong Hew.

REFERENCES

Aravind L, Iyer LM, and Koonin EV. 2006. Comparative genomics and structural biology of the molecular innovations of eukaryotes. Curr Opin Struct Biol. 16: 409–419.

Bonvin AM. 2006. Flexible protein–protein docking. Curr Opin Struct Biol. 16: 194–200.

Booy AT, Haddow JD, Ohlund LB, Hardie DB, and Olafson RW. 2005. Application of isotope coded affinity tag (ICAT) analysis for the identification of differentially expressed proteins following infection of Atlantic salmon (*Salmo salar*) with infectious hematopoietic necrosis virus (IHNV) or *Renibacterium salmoninarum* (BKD). J Proteome Res. 4: 325–334.

Chandonia JM and Brenner SE. 2005. Implications of structural genomics target selection strategies: Pfam5000, whole genome, and random approaches. Proteins. 58: 166–179.

Chen LM, Tran BN, Lin Q, Lim TK, Wang F, and Hew CL. 2008. iTRAQ analysis of Singapore grouper iridovirus infection in a grouper embryonic cell line. J Gen Virol. 89: 2869–2876.

Chen X, Walker AK, Strahler JR, Simon ES, Tomanicek-Volk SL, Nelson BB, Hurley MC, Ernst SA, Williams JA, and Andrews PC. 2006. Organellar proteomics: analysis of pancreatic zymogen granule membranes. Mol Cell Proteomics. 5: 306–312.

Cheung TC, Humphreys IR, Potter KG, Norris PS, Shumway HM, Tran BR, Patterson G, Jean-Jacques R, Yoon M, Spear PG, Murphy KM, Lurain NS, Benedict CA, and Ware CF. 2005. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. Proc Natl Acad Sci USA. 102: 13218–13223.

Eaton HE, Metcalf J, Penny E, Tcherepanov V, Upton C, and Brunetti CR. 2007. Comparative genomic analysis of the family Iridoviridae: re-annotating and

defining the core set of iridovirus genes. Virol J. 4: 11.

Foster LJ, de Hoog CL, Zhang Y, Xie X, Mootha VK, and Mann M. 2006. A mammalian organelle map by protein correlation profiling. Cell. 125: 187–199.

Guerrera IC and Kleiner O. 2005. Application of mass spectrometry in proteomics. Biosci Rep. 25: 71–93.

Holm L and Sander C. 1995. Dali: a network tool for protein structure comparison. Trends Biochem Sci. 20: 478–480.

Huang C, Zhang X, Lin Q, Xu X, Hu Z, and Hew CL. 2002. Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. Mol Cell Proteomics. 1: 223–231.

Khalsa-Moyers G and McDonald WH. 2006. Developments in mass spectrometry for the analysis of complex protein mixtures. Brief Funct Genomic Proteomic. 5: 98–111.

Li Z, Lin Q, Chen J, Wu JL, Lim TK, Loh SS, Tang X, and Hew CL. 2007. Shotgun identification of the structural proteome of shrimp white spot syndrome virus and iTRAQ differentiation of envelope and nucleocapsid subproteomes. Mol Cell Proteomics. 6: 1609–1620.

Lightner DV. 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA.

Liu Y, Wu J, Song J, Sivaraman J, and Hew CL. 2006. Identification of a novel nonstructural protein, VP9, from white spot syndrome virus: its structure reveals a ferredoxin fold with specific metal binding sites. J Virol. 80: 10419–10427.

Namikoshia A, Wu JL, Yamashita T, Nishizawa T, Nishioka T, Arimoto M, and Muroga K. 2004. Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture 229: 25–35.

Otwinowski Z and Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276: 307–326.

Pusey ML, Liu ZJ, Tempel W, Praissman J Lin D, Wang BC, Gavira JA, and Ng JD. 2005. Life in the fast lane for protein crystallization and X-ray crystallography. Prog Biophys Mol Biol. 88: 359–386.

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, and Pappin DJ. 2004. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 3: 1154–1169.

Sample R, Bryan L, Long S, Majji S, Hoskins G, Sinning A, Olivier J, and Chinchar VG. 2007. Inhibition of iridovirus protein synthesis and virus replication by antisense morpholino oligonucleotides targeted to the major capsid protein, the 18 kDa immediate-early protein, and a viral homolog of RNA polymerase II. Virology. 358: 311–320.

Song W, Lin Q, Joshi SB, Lim TK, and Hew CL. 2006. Proteomic studies of the Singapore grouper iridovirus. Mol Cell Proteomics. 5: 256–264.

Song WJ, Qin QW, Qiu J, Huang CH, Wang F, and Hew CL. 2004. Functional genomics analysis of Singapore grouper iridovirus: complete sequence determination and proteomic analysis. J Virol. 78: 12576–12590.

Summerton J and Weller D. 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. 7: 187–195.

Tang X, Wu J, Sivaraman J, and Hew CL. 2007. Crystal structures of major envelope proteins VP26 and VP28 from white spot syndrome virus shed light on their evolutionary relationship. J Virol. 81: 6709–6717.

Tsai JM, Wang HC, Leu JH, Hsiao HH, Wang AH, Kou GH, and Lo CF. 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J Virol. 78: 11360–11370.

van de Braak CB, Botterblom MH, Huisman EA, Rombout JH, and van der Knaap WP. 2002. Preliminary study on haemocyte response to white spot syndrome virus infection in black tiger shrimp *Penaeus monodon*. Dis Aquat Organ. 51: 149–155.

van Hulten MC, Witteveldt J, Snippe M, and Vlak JM. 2001. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology. 285: 228–233.

Wang F, Bi X, Chen LM, and Hew CL. 2008a. ORF018R, a highly abundant virion protein from Singapore grouper iridovirus, is involved in serine/threonine phosphorylation and virion assembly. J Gen Virol. 89: 1169–1178.

Wang HC, Wang HC, Ko TP, Lee YM, Leu JH, Ho CH, Huang WP, Lo CF, and Wang AH. 2008b. White spot syndrome virus protein ICP11: a histone-binding DNA mimic that disrupts nucleosome assembly. Proc Natl Acad Sci USA. 105: 20758–20763.

Wang YC, Lo CF, Chang PS, and Kou GH. 1998. White spot syndrome associated virus (WSSV) infection in cultured and wild decapods in Taiwan. Aquaculture. 164: 221–231.

Williams T, Barbosa-Solomieu V, and Chinchar VG. 2005. A decade of advances in iridovirus research. Adv Virus Res. 65: 173–248.

Wu J, Lin Q, Lim TK, Liu T, and Hew CL. 2007. White spot syndrome virus proteins and differentially expressed host proteins identified in shrimp epithelium by shotgun proteomics and cleavable isotope-coded affinity tag. J Virol. 81: 11681–11689.

Xie X, Xu L, and Yang F. 2006. Proteomic analysis of the major envelope and nucleocapsid proteins of white spot syndrome virus. J Virol. 80: 10615–10623.

Yan X, Olson NH, Van Etten JL, Bergoin M, Rossmann MG, and Baker TS. 2000. Structure and assembly of large lipid-containing dsDNA viruses. Nat Struct Biol. 7: 101–103.

Yang F, He J, Lin X, Li Q, Pan D, Zhang X, and Xu X. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. J Virol. 75: 11811–11820.

Yi G, Wang Z, Qi Y, Yao L, Qian J, and Hu L. 2004. Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J Biochem Mol Biol. 37: 726–734.

Zhang X, Huang C, Tang X, Zhuang Y, and Hew CL. 2004. Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. Proteins. 55: 229–235.

Chapter 10

DNA Vaccines for Viral Diseases of Farmed Fish and Shellfish

Jo-Ann C. Leong, Kristine Romoren, and Oystein Evensen

INTRODUCTION

Global aquaculture has grown rapidly in the last decade and with that growth has come the need for effective disease control measures when the indiscriminant use of antibiotics is not recommended and in some countries, not permitted. Thus, safe and effective vaccines for aquatic animals have become a critical need for the aquaculture industry. Bacterial vaccines made possible the rearing of Atlantic salmon in seawater net-pen cages and virtually revolutionized the production of salmon in the world. The first vaccine for *Listonella anguillarum* was followed quickly by vaccines for *Vibrio ordalii*, *Aeromonas salmonicida*, *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*, and many more for nonsalmonid fish species. The less tractable diseases for vaccine production include the viral and parasitic diseases. This review will concentrate on the vaccines under development for viral diseases of aquaculture fish and shellfish.

There are four basic formulations currently used in viral vaccines: live, attenuated vaccines, whole inactivated (killed) vaccines, purified subunits (proteins or glycoproteins) of the pathogen, and DNA vaccines. There have been reports of viral vaccines using all of these formulations. A live, attenuated viral vaccine for fish was recently made commercially available in Israel against cyprinid herpesvirus 3 (CyHV3) infections in koi carp. The vaccine (KV3) is based on an attenuated strain of the virus that can also be differentiated from the wild CyHV3 as a consequence of genomic deletion in the vaccine strain. Intervet NorBio (now MSD Animal Health) produces a subunit vaccine for infectious pancreatic necrosis virus (IPNV). The vaccine consists of the major IPNV capsid protein produced in bacteria, and is injected with adjuvant into Atlantic salmon (*Salmo salar* L.) prior to their introduction to seawater. This IPNV vaccine remains as the only effective viral vaccine produced by recombinant DNA technology that is commercially available on the world market. Issues with effectiveness and cost have limited the introduction of other viral vaccines using similar technology. Currently, the most promising vaccine formulation for viral diseases is the DNA vaccine consisting of naked plasmid DNA that will drive viral gene expression in the tissues of the vaccinated fish.

Since the first demonstration that intramuscular (i.m.) injection of plasmid DNA encoding a reporter gene could result in the in vivo expression of the encoded protein (Wolff et al. 1990) and the

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

demonstration that DNA encoding a viral protein could generate protective immunity against viral challenge in mice (Ulmer et al. 1993), there has been great interest in using this technology to develop vaccines for aquacultured animals (Leong et al. 1997; Lorenzen and LaPatra 2005; Kurath 2005, 2008). The first indication that the injection of DNA into fish would result in the expression of the encoded gene was reported by Anderson et al. (1996), and the first indication that genetic immunization was possible in fish was reported by the same group in 1996 (Anderson et al. 1996) for infectious hematopoietic necrosis virus (IHNV). These reports were quickly followed by Gomez-Chiarri et al. (1996), who introduced foreign genes into the tissue of live fish by direct injection and particle bombardment, and Lorenzen et al. (1998), who reported protection against viral hemorrhagic septicemia virus (VHSV) by DNA vaccination. Currently, effective DNA vaccination of a large number of fish species against a variety of viral diseases has been demonstrated (Table 10.1), and recently the successful DNA vaccination of shrimp against white spot syndrome virus (WSSV) was reported (Kumar et al. 2008; Rajeshkumar et al. 2009). Despite these reports, only one DNA vaccine for aquacultured fish has been licensed for sale. The Apex-IHN® developed by Aqua Health, Ltd., an affiliate of Novartis, was cleared for marketing by the Canadian Food Inspection Agency on July 15, 2005 (Novartis media release, July 19, 2005; Kutzler and Weiner 2008).

DNA VACCINE DEVELOPMENT CRITERIA

DNA vaccines consist of a bacterial plasmid with a strong promoter, the gene of interest, and a polyadenylation/transcriptional termination sequence (see Section "Vectors"; Fig. 10.1). In most cases, a viral promoter like the immediate early gene promoter and enhancer of human cytomegalovirus (CMV) is used because it is active in a wide variety of tissues. The plasmid DNA also contains its own bacterial DNA replication signals and thus, can be grown in large quantities in bacteria using standard protocols. The DNA is purified, dissolved in saline or phosphate-buffered saline, and administered by injection. Current research is focused on different fish-specific promoters/enhancers, expression of multiple viral genes in sequence for complex viral pathogens, and the design of vectors that would permit the removal of the DNA vaccine-containing cells after immunization.

The criteria that govern the development of a DNA vaccine for viral diseases of aquatic species should include the following:

1. The identification of the immunizing viral antigen as the immunoprotective antigen or antigen(s),
2. The expression of the antigen in the appropriate tissues of the fish,
3. The correct processing of the viral protein so that immunization elicits an immune response to the form of the protein found in the virus,
4. Identification and characterization of the appropriate promoter/enhancer to drive expression in specific tissues for immunization,
5. The duration of expression,
6. The minimal required dose for immunization,
7. The ease of administration to very small fish for some viral diseases,
8. The persistence of the vaccine DNA in the animal,
9. The absence of integration of the vaccine DNA into the genetic material of the injected fish,
10. The efficacy of the vaccine in live virus challenge trials and in field trials,
11. The duration of the immunity, and
12. The demonstration of food safety for the human consumer.

Identification of all serotypes of the viral pathogen must also be determined. These criteria are difficult to meet for many aquatic viral pathogens and require systems where live virus/fish challenge assays are available (Kurath 2008).

For the aquaculture farmer, the best vaccine would be one that is cost-effective, easy to administer, provides 90–100% protection at the right life stage in production, has minimal environmental risk, and leaves no residual problems in the fish species at the time of sale. These are difficult criteria to meet for any vaccine. Companies considering the commercialization of DNA vaccines for aquatic species need to consider all of these factors in their business evaluation.

Table 10.1. Viral DNA Vaccines for Aquacultured Animals.*

Pathogen	Host	Efficacy Trials (>50% RPS)	Viral Gene	Reference
Infectious hematopoietic necrosis virus (IHNV)	Rainbow trout	1 g fish, challenge 3 viral doses 42 days postvaccination (dpv)	G	Anderson et al. 1996
IHNV	Atlantic salmon	57 g, pre-smolt (freshwater) 57 g, smolted (seawater), challenge 56 dpv	G	Traxler et al. 1999
IHNV	Chinook salmon Sockeye salmon Kokanee salmon Rainbow trout	3 g fish, 45 dpv 0.7 and 3 g fish, 35 and 28 dpv 1.7 g fish, 28 dpv 3.2 g fish, 28 dpv	G	Garver et al. 2005
IHNV	Rainbow trout	110 g fish, 42 dpv	G	LaPatra et al. 2000
Viral hemorrhagic septicemia virus (VHSV)	Rainbow trout	13 g fish, 52 dpv (freshwater)	G	Lorenzen et al. 1998
VHSV (marine isolate)	Japanese flounder	3 g fish, 30 dpv 10 g fish, 30 dpv	G	Bryon et al. 2005 Bryon et al. 2006
Hirame rhabdovirus (HIRRV)	Japanese flounder	2 g fish, 28 dpv 3 g fish, 21 dpv 10 g fish, 28 dpv	G Partial G G, N	Takano et al. 2004 Seo et al. 2006 Yasuike et al. 2007
Spring viremia of carp virus (SVCV)	Common carp	11 g fish, 42 dpv	G	Kanellos et al. 2006
SVCV	Koi carp	1.5–4.3 g fish, 28 dpv	G	Emmenegger and Kurath 2008
Infectious pancreatic necrosis virus (IPNV)	Atlantic salmon	20 g fish post-smolts, 60 dpv (not as effective with VP2 alone)	VP2-NS-VP3 VP2 alone	Mikalesen et al. 2004
Infectious salmon anemia virus (ISAV)	Atlantic salmon	20 g fish, pre-smolts, 63 dpv	Hemagglutinin Esterase HE	Mikalsen et al. 2005
Red sea bream iridovirus	Red sea bream	5–10 g fish, 30 dpv	Major capsid protein	Caipang et al. 2006

Table 10.1. (*Continued*)

Pathogen	Host	Efficacy Trials (>50% RPS)	Viral Gene	Reference
Channel catfish virus (CCV)	Channel catfish	4–8 g fish, 28–42 dpv	ORF6 and 59	Nusbaum et al. 2002.
		1 g fish, 35 dpv (not effective)	ORF6 and 59	Harbottle et al. 2005
Atlantic halibut nodavirus (AHNV)	Turbot	3.6 g fish, 35 dpv (early protection)	C, capsid protein	Sommerset et al. 2003
		2.2 g fish, 70 dpv (not effective)	C	Sommerset et al. 2005
IHNV and VHSV	Rainbow trout	2 g fish, 28 dpv (dual protection)	G	Einer-Jensen et al. 2009
Lymphocystis disease virus (LCDV)	Japanese flounder	60–80 g fish, 21 dpv cohabitation	Capsid, 6 kb	Zheng et al. 2006
		60–80 g fish, 77–98 dpv antibody		Tian et al. 2008
White spot syndrome virus	*Penaeus monodon*	7–10 g shrimp, 7, 14, 21, 30 dpv	VP28	Kumar et al. 2008
		8–10 g shrimp, 7, 14, 21, 30 dpv	P28-chitosan	Rajeshkumar et al. 2009

dpv, days postvaccination; rainbow trout, *Oncorhynchus mykiss*; chinook salmon, *Oncorhynchus tshawytscha*; sockeye salmon, *Oncorhynchus nerka*; kokanee salmon, *Oncorhynchus nerka*, land-locked (freshwater); Atlantic salmon, *Salmo salar*; Japanese flounder, *Paralichthys olivaceus*; common carp, *Cyprinus carpio*; red sea bream, *Pagrus major*; Atlantic halibut, *Hippoglossus hippoglossus*; turbot, *Scophthalmus maximus*.

*This table was modified and updated from Kurath (2008).

VACCINE CANDIDATES

Several recent and excellent reviews on DNA vaccines for aquaculture are available (see; Lorenzen and LaPatra 2005; Kurath et al. 2007; Kurath 2008). These reviews provide detailed descriptions of the efficacy trials reported for the fish rhabdoviruses, IHNV and VHSV, both of which are serious pathogens of commercially important salmonid species and for which effective vaccines have been sought for decades. There have also been more than 70 years of research on the rhabdoviral pathogens of salmonid fish species ever since Schaperclaus (1938) described viral hemorrhagic septicemia in rainbow trout, in Europe in 1938 and Rucker et al. (1953) described a disease of possible viral origin in Sacramento River Chinook (1953). The viral proteins for both IHNV and VHSV have been characterized and each viral gene had been cloned and sequenced for many different isolates. Recombinant DNA-derived protein vaccines had already been tested with mixed results (Gilmore et al. 1988; Oberg et al. 1991; Lorenzen and Olesen 1997; Winton 1997) and a large body of evidence was available to indicate that a single viral protein, the viral glycoprotein, was sufficient to immunize against a lethal challenge with live virus (Bootland and Leong 1999; Smail 1999). Moreover, there appeared to be only one major serotype for each virus, although serovariants

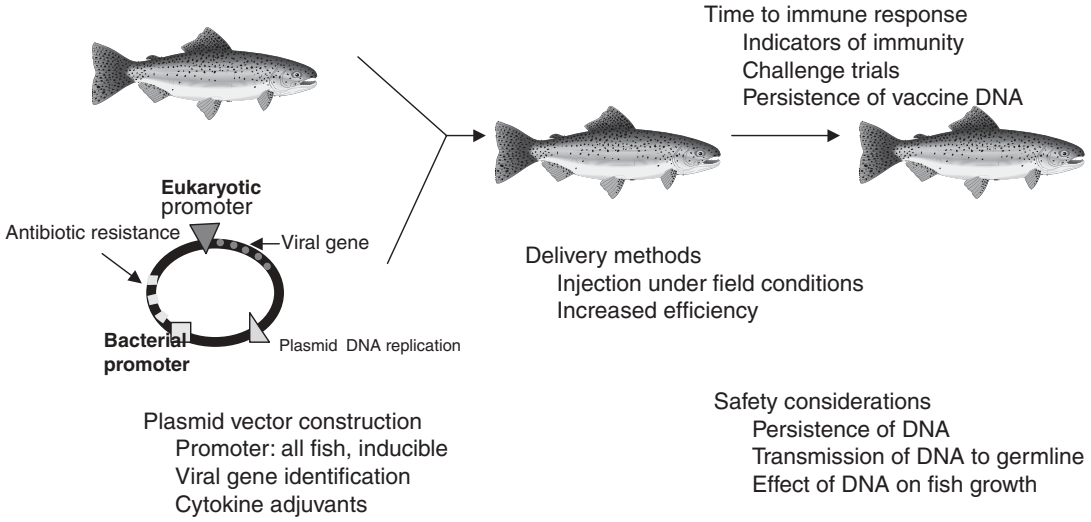


Figure 10.1. Research targets in DNA vaccine development. (For color detail, please see the color plate section.)

had appeared in different fish species and different geographic regions. Laboratory trials had shown that there was sufficient antigenic overlap between the serovariants so that glycoprotein obtained from one virus isolate would induce immunity against several different serovariants (Engelking and Leong 1989a,1989b). Thus, vaccine developers were able to consider developing an effective DNA vaccine for IHNV with a single IHNV G gene and similarly, a single VHSV G gene for the VHSV DNA vaccine. For other rhabdoviral diseases of fish, the viral glycoprotein gene has also proven to be useful in the development of each respective viral DNA vaccine (Table 10.1).

For nonrhabdoviral pathogens, single gene DNA vaccines have not been as efficacious. The DNA vaccine trials for IPNV demonstrate the need for more than just the major capsid protein, VP2. IPNV is a birnavirus whose genome contains two segments of double stranded RNA. The larger RNA segment encodes the major capsid protein, VP2, the minor capsid protein, VP3, and NS, a nonstructural gene encoding a protease responsible for cleaving the polyprotein, VP2-NS-VP3, encoded by segment A. In the Mikalsen et al. (2004) report, the DNA vaccine containing the entire segment A protected the immunized fish against live viral challenge with a relative percent survival (RPS) of 84%. But when VP2 was used alone in the DNA vaccine construction, the RPS was 29%, significantly lower protection. RPS is calculated as (1-cumulative mortality in the vaccinated group/cumulative mortality in the nonvaccinated group) $\times 100$. The relatively poor efficacy of the DNA vaccine encoding VP2 alone was unexpected since recombinant DNA-derived VP2 produced in *Escherichia coli* has proven to be very effective in field trials (Ramstad et al. 2007).

Infectious salmon anemia virus (ISAV) is a very lethal virus of Atlantic salmon, and its appearance in marine-farmed Atlantic salmon has had severe economic consequences in Norway, Faroe Islands (Denmark), the United States, and Canada. The only available vaccine is a whole virus inactivated vaccine that does not fully protect against the virus (Kibenge et al. 2003). Moreover, there remained the problem of persistent virus infection in the vaccinated survivors (McDougall et al. 2001). Vaccination against ISAV is also complicated by antigenic

variation in the hemagglutinin (HA) virion protein, a major immunizing ISAV antigen (Kibenge et al. 2001, 2006) that distinguishes the ISAV isolates into two major serogroups, the European and the North American groups. More recently, Falk et al. (2008) reported the further characterization of these isolates with a panel of monoclonal antibodies that distinguished the European isolates into three serogroups and the North American isolates into six to seven serogroups. DNA-based vaccines for ISAV are under development (Mikalsen et al. 2005), and the early reports indicate that immunization with three doses of plasmid DNA expressing the ISAV segment six encoding the virus HA-esterase protein did reduce the mortality of the fish by 40–60% RPS, as compared with control vaccinated animals receiving plasmid with no insert or plasmid DNA containing the viral nucleoprotein. The authors do report additional trials where only moderate protection was achieved, 29–36% RPS, with single and double immunizations with plasmid DNA. No further reports are available since that 2005 report.

For the betanodaviruses, a group of small RNA viruses with two positive-sense, single-stranded RNA segments in their genome, the development of vaccines is critically important for the successful culture of halibut, turbot, sea bass, Atlantic cod, and grouper. No commercial vaccines are available for the betanodaviruses. However, a recombinant vaccine has been shown to be highly efficacious in young turbot (5 g size) (Sommerset et al. 2005). A DNA vaccine against VHSV offered some protection against nodavirus challenge in turbot, but a DNA vaccine designed specifically for the betanodaviruses did not provide any protection (Sommerset el al. 2003, 2005). Betanodavirus virus-like particles did induce a protective immune response against viral nervous necrosis in European sea bass (Thierry et al. 2006).

DNA vaccines for DNA viruses have had mixed results. The channel catfish virus (IHV-1, ictalurid herpes virus 1), DNA vaccine that induced the expression was reported to be effective in 4–8 g fish at 28–42 days postvaccination by Nusbaum et al. (2002). DNA plasmids encoding genes for IHV-1 open reading frames 6 (membrane protein) and 59 (major membrane glycoprotein) were found to induce protective immunity with 52–100% RPS values for immunized fish. However, Harbottle et al. (2005) reported conflicting results in 1 g channel catfish challenged with CCV at 35 days postvaccination. Although 11 IHV-1 genes, including ORF 6 and 59, were cloned into DNA vaccine expression vectors, none provided protective immunity against channel catfish virus in this study. The difference in age at the time of vaccine introduction may be the reason for the difference in results. In contrast, Caipang et al. (2006) reported a robust immunoprotective response in juvenile red sea bream (5–10 g) against the red sea bream iridovirus vaccinated with a DNA vaccine to the viral major capsid protein. A DNA vaccine to the capsid protein of lymphocystis disease virus (LCDV) was effective in inducing an antibody response in Japanese flounder (Zheng et al. 2006; Tian et al. 2008).

An effective DNA vaccine for WSSV in penaeid shrimp has been long sought by investigators in the shrimp disease field. WSSV is a highly pathogenic virus of penaeid shrimp and is responsible for huge economic losses in the shrimp culture industry worldwide. The virus is a member of the Nimaviridae, belonging to the genus Whispovirus, and has a single double-stranded circular DNA genome of about 300 kbp. When a DNA vaccine constructed to induce the expression of the major capsid protein, VP28, was injected into shrimp (7–10 g weight) intramuscularly and challenged with live virus at 7, 14, 21, and 30 days postvaccination, significant protection was observed on days 7, 14, 21, and 30 with the respective RPS values of 90%, 76.66%, 66.66%, and 56.66%. There was a distinct declining trend in resistance to virus-induced mortality with time after the vaccination. The authors were unable to identify the basis for the immune protection.

VACCINE FORMULATIONS

Vector Construction

The first vectors used for aquaculture DNA vaccines were bacterial plasmids that contained expression cassettes for mammalian cell transcription. Typically, DNA vaccine vectors are closed circular double-stranded DNA molecules that contain a prokaryotic DNA replication origin permitting the production of high copy numbers of the plasmid in bacterial cells and an antibiotic resistance gene

that permits selective growth of bacterial cells containing the vector (Fig. 10.1). The mammalian cell transcription cassette normally carried a promoter element that drove mRNA transcription in eukaryotic cells and sequences that helped to stabilize or enhance translation of the primary transcript. The most widely used promoter/enhancers were derived from the CMV immediate early promoter/enhancer (IEP), a virtually universal promoter/enhancer in mammalian tissues. In fish, the CMV-IEP was very effective. When the pCMV-Luc (plasmid vector driving expression of luciferase) DNA was injected into rainbow trout, luciferase activity was detected in muscle, liver, heart, kidney, stomach, and intestine tissues (Anderson et al. 1996). Similarly, when plasmid DNA containing the reporter gene, green fluorescent protein (GFP) under the control of CMV-IEP was injected into Japanese flounder; GFP was detected in muscle, gill, head kidney, and spleen tissues (Zheng et al. 2006). The CMV-IEP transcription cassette also works in shrimp (*Penaeus monodon*) tissue. Plasmid DNA containing the VP28 gene of WSSV when injected intramuscularly into shrimp drove the expression of VP28 in muscle, gill, head muscle, pleopod, and gut tissues (Kumar et al. 2008). Expression of VP28 was detected with specific antibody.

Long-term persistence of plasmid DNA in injected fish and shellfish is supported by numerous studies. Tonheim et al. (2007) reported the detection of supercoiled plasmid DNA and luciferase expression in Atlantic salmon (*S. salar*) as long as 535 days after injection. In rainbow trout, plasmid DNA has been detected for at least 90 days (Garver et al. 2005) and covalently closed circular plasmid DNA has been detected in trout injected with the IHNV expression plasmid, pCMV-G, for as long as 120 days postinjection (M. Alonso, personal communication). In zebrafish that had received the plasmid DNA, pCMV-GFP, GFP was detected for at least 50 days (Sudha et al. 2001). The fluorescent protein expression was unchanged whether the DNA was introduced by particle bombardment in skin and fin epithelia, skin pigment cells, blood vessel cells, and nerve-like cells or by i.m. injection where the GFP was expressed in muscle, blood vessel cells, dispersed epithelial cells, and neuron-like cells. The expression of luciferase was easily detected at the injection site in muscle tissue in glass catfish (*Kryptopterus bicirrhus*) for at least 2 years (Dijkstra et al. 2001). DNA bombardment of pCMV-CAT (chloramphenicol acetyltransferase) in Japanese flounder produced stable expression of the CAT protein for 60 days in muscle tissue (Tucker et al. 2001).

Optimization of the regulatory elements in the expression plasmid for specific tissue expression and the use of fish-specific regulatory elements are most likely the next step in for researchers seeking more efficient and safe DNA vaccines for aquaculture. An all "fish" DNA vaccine vector is being developed by a number of groups because of concerns regarding the introduction of human viral DNA into food animals. The upstream promoter regions of either rainbow trout interferon (IFN) regulatory factor 1A (IRF-1A) gene or the trout Mx1 gene (Alonso et al. 2003) were tested as potential candidates for trout-specific DNA vaccine plasmid vectors. Neither of these promoters provided the expression levels attained with pCMV-IEP-Luc (expressing luciferase) in chinook salmon cells or carp cells in vitro. However, in rainbow trout, the IRF-1A promoter (pIRF-1A-Luc) provided acceptable levels of expression as compared with pCMV-IEP-Luc, and in immunization trials, pIRF-1A-G (expressing the IHNV glycoprotein) induced protection equivalent to that of pCMV-IEP-G.

The carp beta-actin promoter was evaluated by Gomez-Chiarri and Chiaverini (1999) in Atlantic salmon using a luciferase reporter gene. This promoter compared well with the CMV-IEP that appeared to be twice as effective in producing luciferase activity in the muscle tissue at 8 days postinjection. A second promoter, the lactate dehydrogenase (Ldh), from *Fundulus heteroclitus* was also tested and found to be not as effective.

There are a number of other fish promoters that are possible candidates for DNA vaccine vector construction. They include the regulatory sequences for genes encoding the metallothionein A and B, protamine, insulin, insulin-like growth factor II, growth hormone, prolactin, gonadotropin, gonadotropin-releasing hormone, albumin, transferrin, estrogen receptor, and the heat-shock-like protein. Most recently, Ruiz et al. (2008) examined the following promoters for their potential in the construction of an all "fish vector" for a VHSV DNA vaccine: an

altered version of the CMV-IEP, MCV1.4; an altered version of the carp beta-actin promoter, AE6; the long terminal repeats (LTR) of zebrafish and walleye retroviruses; trout Mx1 promoter; carp myosin-heavy-chain promoter; flatfish pleuronectin promoters; and the salmonid sleeping beauty /medaka Tol2 transposon repeats. They conducted their assay in EPC cells and monitored expression by flow cytometry to detect cells expressing VHSV-glycoprotein and stained with a monoclonal antibody to the viral glycoprotein. The best promoters in EPC cells as judged by the number of G-expressing cells and the average G-expression level per cells included MCV1.4, the AE6, LTRz, LTRw, and Mx. Furthermore, the AE6 and LTRz promoters reached a ∼3–5-fold higher average G-protein expression per cell than the rest of the tested promoters. Thus, the AE6 and/or LTRz promoters might well be appropriate fish substitutes for the human IE-CMV (MCV1.4 version) promoter for in vivo fish vaccination.

The in vivo testing of the carp beta-actin promoter was recently reported (Chico et al. 2009) in a comparison testing of the immunity induced to VHSV. Two vaccine plasmids were tested, pAE6-Gvhsv and pMCV1.4-Gvhsv. The study revealed some interesting findings. (1) The MCV1.4 promoter induced an earlier RNA response in transfected EPC cells with ten times the G-RNA expression as compared with pAE6-Gvhsv expression at day 2, but by day 5, pAE6-promoted expression surpassed the RNA expression of pMCV1.4-Gvhsv by tenfold. (2) In muscle tissue of DNA vaccinated fish, however, the opposite was true, i.e., expression at the RNA and protein level was greater for pAE6-Gvhsv. (3) Antibody levels induced in vaccinated fish were significantly higher for the pAE6-Gvhsv vaccinated fish. This antibody detected by ELISA, however, had very little virus neutralization activity. (4) When the vaccinated fish were challenged with live virus at 30 days postvaccination, both DNA vaccines provided the same level of protection. (5) Quantitative PCR measurements of 20 different immune response genes in muscle, head kidney, and spleen at 3 and 10 days postimmunization provided some indication that the type I IFN-related immune response is correlated with the levels of Gvhsv expression and that the proinflammatory cytokine genes were upregulated in the muscle of the pAE6-Gvhsv immunized fish. In summary, the carp beta-actin promoter is an acceptable fish promoter for DNA vaccines in fish.

CYTOKINES AS ADJUVANTS FOR DNA VACCINES

The induction of acquired immunity in fish vaccination requires the stimulation of strong and long-lasting memory T-cell responses (for review, see Secombes 2008). In its simplest description, the immunization process involves the uptake of a viral protein, for example, by macrophage/dendritic cells and the processing of the protein into antigenic fragments that are presented by the macrophage/dendritic cell on its surface to T-helper lymphocytes. When the T-helper cell "recognizes" the antigenic fragment and binds to the antigen-presenting dendritic cell, a number of stimulatory molecules are produced including interleukin (IL)-1 and tumor necrosis factor (TNF) by the dendritic cell and IL-2 and IFN-γ by the T-helper lymphocyte. These "cytokines" act to stimulate the proliferation of T-helper lymphocytes and T-cytotoxic lymphocytes. The T-helper cells stimulate B cells to produce specific antibody and the cytotoxic T cells destroy virus-infected host cells. Finally, when the virus infection is contained, these immune response cells regress and form a pool of "memory" cells. Vaccination is aimed at forming this pool of memory cells so that the next exposure to the virus is met with a quicker and larger immune response. Vaccines have been formulated to increase the memory cells by adding soluble cytokines to the DNA vaccine procedure, adding a separate plasmid encoding a cytokine gene to the vaccine formulation, or by adding a cytokine gene to the DNA vaccine plasmid itself (Leong et al. 2000; Jimenez et al. 2006).

In mammals, different cytokines have proven to be efficacious as adjuvants for DNA vaccination (for review, see Liu et al. 2006). Granulocyte–macrophage colony stimulating factor (GM-CSF) enhanced the immune response to DNA vaccines as has IL-1β, IL-2, IL-7, IL-12, IL-15, and TNF-α. Chemokines, i.e., cytokines with chemoattractant activity, have also been found to enhance the DNA vaccines against viral pathogens in mammals. The tested chemokines included IL-8, macrophage inflammatory protein (MIP-1α), and CCL-21.

In fish, there is evidence for type I (α, β) and type II (γ) IFN genes, including fugu (*Takifugu rubripes*), common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*S. salar*), and rainbow trout (*Oncorhynchus mykiss*) (Altman et al. 2003; Robertsen et al. 2003; Long et al. 2004; Zou et al. 2004, 2005, 2007; Milev-Milovanovic et al. 2006; Levraud et al. 2007; Stolte et al. 2008). In rainbow trout, three type I IFN genes (rtIFN1, rtIFN2, and rtIFN3) and two type II IFN genes (IFN-γ1 and IFN-γ2) have been identified (Purcell et al. 2009). Some of these genes have been used to augment the effectiveness of DNA vaccines in fish (Secombes 2008).

Jimenez et al. (2006) has shown that coadministration of a plasmid encoding the rainbow trout chemokine, IL-8, with a DNA vaccine for VHSV, increases the expression of the proinflammatory cytokines such as IL-1β and TNF-α. These investigators have more recently reported that coadministration of IL-8 with the DNA vaccine for VHSV resulted in the increased expression of all CC chemokines, CK-5A, CK-6, CK-7A, CK-7B, and in particular CK-5B. These studies suggest that IL8 might be an effective molecular adjuvant for DNA vaccination in fish.

DELIVERY METHODS

Intradermal and Intramuscular Injection

DNA vaccines against IHNV and VHSV are delivered by the i.m. route, and there are few if any controlled studies on the use of intradermal delivery of DNA vaccines to fish. The i.m. route is rarely used for administration of fish vaccines in general as there is a possibility of creating unsightly scaring and leakage of the inoculum after injection. Further, inclusion of oil- or alum-based adjuvants for improved immunogenicity may cause extensive muscle necrosis that often ulcerates (Horne and Ellis 1988). Thus, the i.p. route of delivery is the most commonly used administration route for injection vaccines for fish. However, neither route is suitable for small fish as the automated vaccination devices are made for fish above a certain size, typically >35 g (Ellis 1988; Horne and Ellis 1988). Both IHN and VHS affect fish at an early life stage, and the younger the fish, the higher the disease susceptibility (Leong et al. 1988; de Kinkelin 1988). Thus, exploration of other routes of delivery is required.

Gene Transfer Inefficiency

DNA, which is a large hydrophilic molecule with an overall negative charge, has to enter the cell and reach the nucleus for translation into RNA, with subsequent transcription into encoded protein. Barrier effects of lipid membranes and diffusion barriers in the cell prevent a passive transportation (Pouton and Seymour 2001). An efficient gene delivery systems will complex and condense DNA molecules through charge interactions and interact with cell membranes. This will trigger uptake of the complexed DNA and result in delivery across the cellular membrane. A gene delivery system may also protect the plasmid from degradation by nucleases in the extracellular biological milieu (Walter et al. 2001). As already mentioned, the i.m. route of delivery is successful for the delivery of naked DNA. Nevertheless, the overall efficiency of transfection is usually low and about 1% of the cells are usually transfected in mammals (Pouton and Seymour 2001). For other routes of delivery, the transfection efficiency may be even lower. The use of gene carriers frequently applied for transfection of cells in vivo and in vitro in mammals has not fully been explored in fish and may increase the transfection efficiency.

Formulating Plasmid DNA

The large, negatively charged hydrophilic DNA molecule must enter the cell and reach the nucleus before translation and transcription can occur. The size and the charge of the molecule are drawbacks for an efficient delivery to the cells. A number of delivery systems have therefore been applied to transfect cells both in vivo and in vitro. The ideal gene carrier should have the following properties: (a) the carrier must bind to DNA to protect it from degradation and also condense the DNA; (b) the complex must be stable enough to reach the cells and should preferably be cell- or tissue-specific to ensure transfection at the desired location; (c) the complex must facilitate entry into cells and endosomal escape; and (d) it should be nontoxic, and easy to produce in large quantity (Gao and Huang 1995; Thierry and Sullivan 1997; van de Wetering 1999). Advantages and disadvantages of physical delivery methods,

Table 10.2. Advantages and Disadvantages of Different Gene Delivery Methods Used for DNA Vaccines (van de Wetering 1999; Prasmickaite 2002; Felgner 1997). Properties Being Advantageous for DNA Vaccines May be Disadvantageous for Gene Therapy.

Gene Delivery methods		Advantages	Disadvantages
Physical methods	Microinjection, ultrasound, electroporation, particle bombardment (gene gun)	High transfection rates	Sophisticated/expensive techniques, high mortality rate of cells
Viral carriers	Retrovirus, adenovirus, lentivirus, adeno-associated virus, herpex simplex virus	High efficiency, transducer dividing and nondividing cells	Not tissue specific, risk for infecting germ cells, strong immune and inflammatory responses directed against the vector, difficult large-scale production, size of plasmid limited
Bacterial carriers	Salmonella, Shigella, or Listeria spp.	Targeting and ability to survive in APCs, immunostimulatory activity, oral and mucosal administration	Plasmid integration into host genome, complex large-scale production
Nonviral carriers	Naked DNA	Relatively easy to manufacture	Large size and negative charge, unsuitable for targeting, degradation by nucleases in vivo
	Calcium phosphate precipitation	Simple, cheap, low toxicity	For ex vivo use, low transfection efficiency
	Liposomes and polymers (e.g., chitosan, DEAE-dextran, and PEI)	Nonrestrictive in the size of DNA to be delivered, passive/active targeting, biodegradable, easy manufacturing	Not as efficient as viral vectors, toxicity may be observed for some systems, nonoptimal intracellular trafficking

viral, bacterial and nonviral carriers are summarized in Table 10.2.

Liposomes

Liposomes are colloidal lipid vesicles formed when amphipathic lipids are exposed to an aqueous environment. Variations in liposome composition, e.g., charged or neutral lipids, saturated/unsaturated lipids or presence of cholesterol, and preparation methods, e.g., mechanical methods, methods based on detergent removal can determine the type of liposomes formed, often referred to as multilamellar, multivesicular or unilamellar liposomes, large unilamellar vesicles, and small unilamellar vesicles. This will also influence the properties, e.g., size, zetapotential of the liposomes. There are several advances of liposomal drug formulations (Allen

1998). They are solubility enhancers; they may protect the drug from degradation; sustained release can be achieved; and they may alter the pharmacokinetics and biodistribution of the drug. Today, liposomes are used for a variety of medical purposes, e.g., in anticancer therapy, in the treatment of infectious diseases, in vaccines, and in imaging (reviewed among others by Crommelin and Schreier (1998).

Scientists have been using conventional liposomes for gene delivery since the beginning of the 80s (Fraley et al. 1980, 1981). However, these types of liposomes were of limited efficiency until Behr demonstrated that cationic liposomes can complex and condense DNA (Behr 1986) and Felgner and co-worker introduced the transfection reagent Lipofectin® (Felgner et al. 1987). Since then their use in gene delivery has escalated. The mechanisms include simple electrostatic interactions between the cationic lipid and the phosphate groups of DNA (Zuidam and Barenholz 1998) and the cationic liposome-DNA complex is called lipoplex (Felgner et al. 1997). Cationic liposome formulations mediate transfection through amphipathic and fusogenic properties. Amphipathic molecules interact with the negatively charged phosphate backbone of DNA, thereby neutralizing the charge of the molecule. This will promote condensation of DNA into a more compact structure. The fusogenic properties are often mediated by the helper lipid DOPE that can induce fusion and/or destabilization of the plasma membrane and facilitate the intracellular release of complexed DNA (Mahato et al. 1997; Pedroso de Lima et al. 2001; Zhdanov et al. 2002). The focus of liposome-based transfection has been variation in the chemical structure of the lipids and choice of neutral helper-lipids, the mechanism of DNA delivery and in vivo applications of the lipoplexes.

Mechanism of Cationic Lipid-based DNA Delivery

There is a general agreement that delivery to intracellular compartments requires binding of the complex to the cell, internalization, endosomal escape, and nuclear localization (Smith et al. 1997). The lead theory claims that the mechanism of uptake is endocytosis, although direct fusion with the cell membrane is also a possible mechanism (Bally et al. 1999; Harashima et al. 2001; Zhdanov et al. 2002; Wiethoff and Middaugh 2003). Once internalized, the intracellular vesicles carrying the vectors fuse with organelles collectively referred to as the endocytic compartment. Endosomal escape is necessary for efficient gene delivery and is thought to involve lipid mixing between the endosome and cationic lipid membranes, which lead to disruption and release of DNA into the cytoplasm. However, the exact mechanism is not defined and can vary between different cells and lipid delivery system (Szoka et al. 1996; Wiethoff and Middaugh 2003). After endosomal escape, DNA must enter the nucleus. It is still not clear how the DNA plasmid is translocated from the cytoplasm into the nucleus during cationic lipid-mediated delivery. At least three possibilities exist. The DNA can pass into the nucleus through nuclear pores; it can physically associate with chromatin during mitosis when the nuclear envelope breaks down; or it could transverse the nuclear envelope (Bally et al. 1999; Wiethoff and Middaugh 2003). Of these three possibilities, the first two seem most likely (Wiethoff and Middaugh 2003).

Use of Liposome-Formulations in Fish

There are relatively few studies addressing the use of liposomal-based delivery systems in fish. Clodronate liposomes have been shown to deplete macrophage subpopulations after intravenous delivery to rainbow trout and are a useful model for the investigation of piscine macrophage functions (Espenes et al. 1997). There are examples of liposomes formulated with LPS from *A. salmonicida*, generating an antibody response to LPS that lasts several weeks longer than immunization with LPS alone (Nakhla et al. 1994, 1997). Delivery of DNA to rainbow trout using positively charged liposomes improved uptake into fin cells, especially when combined with ultrasound pulses (Fernandez-Alonso et al. 1999, 2001). Others have found that liposomal (cationic) delivery by immersion to rainbow trout results in formation of large liposomal–mucin complexes on gill epithelia with lethal effects (Romøren et al. 2002). Furthermore, the distribution of neutral, negatively and positively charged liposomes are investigated following various routes of administration (Power et al. 1990; Nakhla et al. 1997; Romøren et al. 2004).

THE RESPONSE OF THE IMMUNIZED HOST

Although the efficacy of DNA vaccines against many viral diseases of fish has been studied (Table 10.1), the immune response following DNA vaccination is best known for vaccines against virus infections of the genus *Novirhabdovirus*, particularly VHSV and IHNV. The summary of the host immune response to DNA vaccination will thus focus on what has been published for these two viruses.

The immune response following DNA vaccination against IHN and VHS has been separated into three distinct and interrelated phases: the early, systemic, and late antiviral response (LAVR) (Kurath 2005; Lorenzen and LaPatra 2005). In addition to temporal separation, there is also a spatial segregation of the response. First, there is an innate response in the injected muscle (Lorenzen et al. 2000; LaPatra et al. 2001; Lorenzen et al. 2002a), which is followed by a systemic involvement of kidney and spleen, possibly also in the gills. These responses (local and systemic) are elicited weeks prior to antibodies can be measured in serum (Lorenzen et al. 2002b) and are collectively described as the early antiviral response (EAVR). This is followed by a systemic antiviral response (SAVR) that is a specific immune response. At later stages, there is a transition into a LAVR where the immune mechanisms involved are less well understood.

Injection-site EAVR

Boudinot et al. (1998) were the first to study expression of host genes at the injection site following VHSV and IHNV DNA vaccine delivery. They observed an increased expression of Mx mRNA and major histocompatibility complex (MHC) class II genes by reverse transcription-polymerase chain reaction (RT-PCR) at the site of injection. In a similar study, Takano et al. (2004) showed MHC and T-cell receptor mRNA upregulation in muscle tissue of Japanese flounder as early as 1 day and after vaccination using a DNA vaccine against hirame rhabdovirus. Boudinot et al. (1998) also showed expression of viral G protein at the site of injection and later Lorenzen et al. (2005) performed in situ staining of cells involved in local reactions at the injection site. Staining for Ig+ cells, complement factor 3 (C3) and MHC II positive cells was shown by immunofluorescence in close proximity to muscle cells expressing VHSV G protein. It has to be taken into account that in situ studies require injection of relatively high doses of DNA, typically around 20 μg DNA per fish, compared with what is used for standard vaccination and challenge studies (0.5–1 μg per fish). Analyses of transcriptome profiles by microarray from the injection site of rainbow trout identified markers of antigen-presenting cells and leukocytes, and also soluble inflammatory markers (Purcell et al. 2006). Innate response parameters also included type I and II IFN pathways (Purcell et al. 2006). These studies all show that there is a strong inflammatory response at the site of injection associated with expression of the foreign protein on the surface of the muscle cell/myocyte (Lorenzen et al. 2005; Einer-Jensen et al. 2009).

Systemic EAVR

The early systemic immune responses to DNA vaccination were described by Kim et al. (2000) in an IHN DNA vaccine study, where they showed upregulation of Mx protein in kidney and liver at early time (days) postvaccination. Transcript profiling by microarray showed comparable results from head kidney tissue of DNA vaccinated Japanese flounder, where IFN-induced genes included Mx-1 and an IFN regulatory factor, plus nonspecific immune genes, 1 and 3 days postvaccination (Bryon et al. 2005, 2006). Gene transcripts of viral-inducible genes (Vig) have been found upregulated by RT-PCR in liver, spleen, and kidney 7–10 days postvaccination (Boudinot et al. 1999, 2001; Kim et al. 2000; McLauchlan et al. 2003; Purcell et al. 2004, 2006; Acosta et al. 2005, 2006; Sanchez et al. 2007; Tafalla et al. 2007).

From the initial studies, it was clear that EAVR responses were systemic but occurred too early (postvaccination) for adaptive immunity to be involved, particularly in cold-water fish. Most of these initial studies were performed at water temperatures around 10°C. Later studies have focused on the importance of water temperature and has shown that fish kept at 5°C will still be protected against VHSV challenge at 8 days postvaccination, although no Mx expression (mRNA) was measured in the liver in these fish (Lorenzen et al. 2009). Lower temperatures prolong the duration of EAVR measured

by Mx expression levels in liver compared with fish kept at higher temperatures (10°C or 15°C) (Lorenzen et al. 2009). It is thus conceivable that DNA vaccine-induced early antiviral responses are nonspecific and transient, and cross-protect against heterologous virus species on the basis of IFN-related or IFN-induced responses (Lorenzen et al. 2002a). In line with this observation, a VHS G-protein-based DNA vaccine provides early protection in turbot (*Scophthalmus maximus*) against nodavirus challenge (Sommerset et al. 2003). These responses do not correlate with immunostimulatory properties of the vector backbone, while it correlates well with G protein synthesis of IHNV DNA vaccines (Garver et al. 2006).

Collectively these findings indicate that systemic involvement of the type I IFN and IFN-induced genes is part of the EAVR and it is not entirely based on a response at the injection site. Systemic involvement in terms of gene expression is more distinct in kidney and spleen compared with the gills. A detailed understanding of the kinetics and relative importance of local and systemic EAVR following DNA vaccination in fish has to await more research.

Systemic Antiviral Responses

The SAVR induced by IHN and VHS is characterized by specific protection against the corresponding pathogen and the cross-protection against heterologous challenge with IHNV and VHSV is no longer seen. SAVR often involve an antibody response that has been studied for IHN (LaPatra et al. 1993; Boudinot et al. 1998) and VHS (Bernard et al. 1983; Boudinot et al. 1998; Lorenzen and LaPatra 1999), and is also seen for DNA vaccines against lymphocystis virus (Tian et al. 2008). Circulating neutralizing antibodies are found as early as 23 days postvaccination against IHN and VHS, however, only in a few fish of those given one injection (Boudinot et al. 1998). After boost-injection (23 days postprime) all fish were found with neutralizing antibody titers (Boudinot et al. 1998). For IHN, it was also been shown that passive transfer of immune serum from survivors to 1 g rainbow trout fry confer resistance toward lethal challenge (LaPatra et al. 1993). It should be added that protection against lethal challenge has been observed repeatedly without circulating, neutralizing antibodies being detected (Lorenzen et al. 1998; Kim et al. 2000; LaPatra et al. 2000; Kurath et al. 2006).

The importance of cell-mediated immune responses following DNA vaccination has been studied for rainbow trout vaccinated with a VHSV-G DNA vaccine (Utke et al. 2008). It was shown that peripheral blood leukocytes killed MHC-I matched RTG-2 cells and also VHSV-infected xenogeneic cells. IHNV-infected target cells were not killed indicating specific killing (Utke et al. 2008). The fact that MHC-I matched and xenogeneic cells were killed would indicate involvement of cytotoxic cells and also natural killer cells.

Late Antiviral Responses

The immune mechanisms of the LAVRs have not been studied in any detail. It has been shown that efficacy wane with time postvaccination but high levels of protection can still be observed up to 5–6 months for fish vaccinated against VHS (Lorenzen et al. 2000). Further, rainbow trout vaccinated against IHN are protected up to 2 years postvaccination (Kurath et al. 2006) but relative protection is usually lower compared with what is found during the SAVR phase. Rainbow trout vaccinated with VHSV G protein encoding DNA vaccines are protected against lethal challenge at 9 months postvaccination, but at such late stage, postvaccination have no neutralizing antibodies (McLauchlan et al. 2003). The same group of fish was found positive for neutralizing antibodies at 8 weeks and 6 months postvaccination. It should also be added that level of antibodies at different time postvaccination in DNA vaccinated fish depends on vaccine dose and also water temperature.

REGULATORY CONSTRAINTS

Safety concerns regarding the use of DNA vaccines include the risk of integration of the plasmid into the host genome, induction of anti-DNA antibodies, and autoimmunity (Pisetsky 1995; Webster and Robinson 1997). Of concern is also the source of DNA incorporated into the vector, e.g., the promoter/enhancer regions and the antibiotic resistance markers (FDA 1996 The European Agency 1998, 2001). Regulatory agencies in the United States and Europe are, therefore, moving quickly to put into place guidelines for the manufacture and licensing

of DNA vaccines (FDA 1996). To address the issues of safety, FDA has published a list of guidelines to consider in the design and optimization of DNA vaccines (FDA 1996). These guidelines recommend avoidance of (i) strong homology between plasmid DNA and human DNA sequences, (ii) eukaryotic or viral promoters/enhancers and termination/polyadenylation sequences, and (iii) genes encoding β-lactam antibiotics as a selection marker. Although concerns about the safety of DNA vaccines are different for food source animals (Simoes et al. 1999), the FDA list of safety issues must also be taken into consideration for DNA vaccines for fish.

The fate of DNA being administrated to fish can be determined by PCR analysis. In goldfish, no distribution or integration of the foreign DNA was detected following i.m. injection of naked DNA (Kanellos et al. 1999a). Nevertheless, this may be different in other fish species. The CMV promoter originates from a human pathogen, and fish vaccines containing this genetic sequence are considered "unsafe" to federal licensing agents. Many experiments with reporter- and viral genes are performed with such "unsafe" promoters in fish, like the CMV (Anderson et al. 1996; Gomez-Chiarri et al. 1996; Fernandez-Alonso et al. 1997, 2001; Russell et al. 1998; Kanellos et al. 1999a, b; Lee et al. 2000; Torgersen et al. 2000; Tucker et al. 2001), SV40 (Hansen et al. 1991; Tucker et al. 2001), and CMVtk-promoters (Gomez-Chiarri and Chiaverini 1999). Plasmids with fish-derived promoters have, therefore, been under investigation; examples are the carp β-actin promoter (Rahman and Maclean 1992; Anderson et al. 1996; Gomez-Chiarri and Chiaverini 1999), the killifish *F. heteroclitus* lactate dehydrogenase B promoter (Gomez-Chiarri and Chiaverini 1999), the rainbow trout Mx1 and IFN regulatory factor 1A promoters (Alonso et al. 2003), the sockeye salmon gonadotropin-releasing hormone promoter (Coe et al. 1995), and the rainbow trout metallothionein-B gene promoter (Samson et al. 2001). Some of these promoters mediate gene expression at the same levels as the CMV promoter, others are less effective or not tested in vivo. The concerns that have been raised regarding the use of genes encoding ampicillin and β-lactam antibiotics as a selection markers may be avoided by the use of antibiotics like kanamycin or neomycin (FDA 1996).

Persistence and Distribution of Plasmid DNA

Distribution and persistence of plasmid DNA in Atlantic salmon was studied by Tonheim et al. (2007). They injected 100 microgram of plasmid DNA dissolved in phosphate buffered saline into Atlantic salmon of a mean weight 70 g. The fish were in freshwater at a temperature of 10–12°C. Following i.m. injection samples were obtained from blood and different tissues and organs up to almost 1 year after administration (535 days after injection). By the use of Southern blotting techniques, open circular and supercoiled plasmid DNA were found at the injection site. Fragments of plasmid were also found in kidney up to day 535 postinjection by real-time PCR methods. Interestingly, luciferase transcripts and activity were identified at the injection site up to day 535 postinjection; however, analyzing the methylation pattern findings suggested that the plasmid DNA did not replicate in vivo. These findings are indicative that plasmid DNA can persist for a prolonged time after i.m. injection at the injection site (Tonheim et al. 2008).

PUBLIC OPINION

As mentioned by Leitner (2001), the idea of gene transfer raises irrational concern in many people who do not realise that we are under constant attack by viruses, bacteria, and parasites, which force their genes into our cells and use them as factories to produce their progeny. Many will also misunderstand the term genetic immunization with gene therapy. A patient dying during a gene therapy trial was widely publicized in the media (Somia and Verma 2000; Zallen 2000). In Norway, the Norwegian Biotechnology Advisory Board still considers animals as genetically modified organisms if they are vaccinated with DNA vaccines, although this attitude is being challenged by the scientific community and the board is in the process of modifying their statements. As an example, a chronicle in one of the lead newspapers in Norway, Aftenposten claimed that fish injected with DNA vaccines are genetically modified (Moy 1999). It all boils down to a biosafety issue and it will require great communication skills from the scientists and the scientific community to convince people in general as well as the decision-makers within governmental bodies

and licensing authorities that the DNA technology is safe and will pose no hazard to the consumer. Despite the potential this technology holds, the public needs to accept and trust it before it can be taken into general use.

CONCLUSIONS AND FUTURE DIRECTIONS

DNA vaccine development for fish diseases, particular viral pathogens, have made major advances in the last decade, culminating with the approval of the first fish DNA vaccine for IHNV in salmon. A number of technologies aimed at increasing the potency of DNA vaccines are under evaluation in laboratory and field trials. These include improvements in the vectors themselves, the codelivery of cytokines, alternative delivery technologies, and co-induction of innate immunity. Criticisms that the cost for development of these vaccines are prohibitive but a counter argument can be made that DNA vaccines may be the only alternative for many diseases, especially viral diseases, of fish. Destruction of infected stocks of fish and sterilization of the environment when there is an outbreak is not always possible and desirable when the threatened fish stock is important ecologically and genetically. Thus, continued development of these vaccines is critically important to the aquaculture industry.

REFERENCES

Acosta F, Collet B, Lorenzen N, and Ellis AE. 2006. Expression of the glycoprotein of viral haemorrhagic septicaemia virus (VHSV) on the surface of the fish cell line RTG-P1 induces type 1 interferon expression in neighbouring cells. Fish Shellfish Immunol. 21: 272–278.

Acosta F, Petrie A, Lockhart K, Lorenzen N, and Ellis AE. 2005. Kinetics of Mx expression in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar* L.) parr in response to VHS-DNA vaccination. Fish Shellfish Immunol. 18: 81–89.

Allen TM. 1998. Liposomal drug formulations. Rationale for development and what we can expect for the future. Drugs. 56: 747–756.

Alonso M, Johnson M, Simons B, and Leong JA. 2003. A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. Vaccine. 21: 1591–1600.

Altman SM, Mellon MT, Distel DL, and Kim CH. 2003. Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. J Virol. 77: 1992–2002.

Anderson ED, Mourich DV, Fahrenkrug SC, La Patra SC, Shepard J, and Leong JC. 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol Mar Biol Biotech. 5(2): 114–122.

Anderson ED, Mourich DV, and Leong JC. 1996. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Mol Mar Biol Biotech. 5(2): 105–113.

Bally MB, Harvie P, Wong FMP, Kong S, Wasen EK, Reimer DL. 1999. Biological barriers to cellular delivery of lipid-based DNA carriers. Adv Drug Deliv Rev. 38: 291–315.

Behr JP. 1986. DNA strongly binds to micelles and vesicles containing lipopolyamines or lipointercalants. Tetrahedron Lett. 27: 5861–5864.

Bernard J, de Kinkelin P, and Bearzotti-le Berre M. 1983. Viral hemorrhagic septicemia of rainbow trout: relation between the G polypeptide and antibody production in protection of fish after infection with the F25 attenuated variant. Infect Immunity. 39: 7–14.

Bootland LM and Leong JC. 1999. Infectious haematopoietic necrosis virus. In: Fish Diseases and Disorders (eds. Woo PTK and Bruno DW). *CAB International*, Wallingford. pp 57–121.

Boudinot P, Blanco M, de Kinkelin P, and Benmansour A. 1998. Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific immunity and nonspecific responses in rainbow trout. Virol. 249: 297–306.

Boudinot P, Massin P, Blanco M, Riffault S, and Benmansour A. 1999. Vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family. J Virol. 73: 1846–1852.

Boudinot P, Salhi S, Blanco M, and Benmansour A. 2001. Viral haemorrhagic septicaemia virus induces vig-2, a new interferon-responsive gene in rainbow trout. Fish Shellfish Immunol. 11: 383–397.

Bryon JY, Ohira T, Hirono I, and Aoki T. 2005. Use of cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol. 18(2): 135–147.

Bryon JY, Ohira T, Hirono I, and Aoki T. 2006. Comparative immune responses in Japanese flounder, Paralichthys olivaceus after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant

glycoprotein and DNA vaccine using a microarray analysis. Vaccine. 24: 921–930.

Caipang CMA, Takano T, Hirono I, and Aoki T. 2006. Genetic vaccines protect red seabream, *Pagrus major*, upon challenge with red seabream iridovirus (RSIV). Fish Shellfish Immunol. 21: 130–138.

Chico V, Ortega-Villaizan M, Falco A, Tafalla C, Perez L, Coll JM, and Estepa A. 2009. The immunogenicity of viral haemorragic septcaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences. Vaccine. 27: 1938–1948.

Coe IR, von Schalburg KR, and Sherwood NM. 1995. Characterization of the Pacific salmon gonadotropin-releasing hormone gene, copy number and transcription start site. Mol Cell Endocrinol. 115: 113–122.

Crommelin DJA and Schreier H. 1998. Liposomes. In: Colloidal drug delivery systems (ed. Kreuter J). Marcel Dekker New York. pp 73–190.

de Kinkelin P. 1988. Vaccination against viral haemorrhagic seticaemia. In: *Fish Vaccination* (ed. Ellis AE). 1st ed. Academic Press, San Diego, CA. pp 172–192.

Dijkstra JM, Okamoto H, Ototake M, and Nakanishi T. 2001. Luciferase expression 2 years after DNA injection in glass catfish (*Kryptopterus bicirrhus*). Fish Shellfish Immunol. 11: 199–202.

Einer-Jensen K, Delgado L, Lorenzen E, Bovo G, Evensen O, LaPatra S, and Lorenzen N. 2009. Dual DNA vaccination of rainbow trout (*Onchorhynchus mykiss*) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection. Vaccine. 27: 1248–1253.

Ellis AE. 1988. Ontogeny of the immune system on teleost fish. In: Fish Vaccination (ed. Ellis AE). 1st ed. Academic Press, San Diego, CA. pp 20–31.

Emmenegger EJ and Kurath G. 2008. DNA vaccine protects ornamental koi (*Cyprinus carpio* koi) against North American spring viremia of carp virus. Vaccine. 26(50): 6415–6421.

Engelking HM and Leong JC. 1989a. The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. Virus Res. 3: 213–230.

Engelking HM and Leong JC. 1989b. Glycoprotein from infectious hematopoietic necrosis virus (IHNV) induces protective immunity against five IHNV types. J Aquat Anim Health. 1: 291–300.

Espenes A, Press CMcL, Van Rooijgen N, and Landsverk T. 1997. Apoptosis in phagocytotic cells of lymphoid tissues in rainbow trout (*Oncorhynchus mykiss*) following administration of clodronate liposomes. Cell Tissue Res. 289: 323–331.

Falk K, Wiik-Nielsen J, and Cloutier SC. 2008. Serotyping of ISAV using monoclonal antibodies. Abstract. 2008 Fish Health Section-American Fisheries Society Annual Meeting, Charlottetown, Prince Edward Island, Canada, July 9–12, 2008.

Food and Drug Administration (FDA). 1996. Points to consider on plasmid DNA vaccines for preventive infectious disease indications. Center for Biologics Evaluation and Research, Office of Vaccine Research and Review. www.fda.gov/cber/gdlns/plasmid.pdf 1–30.

Felgner PL. 1997. Nonviral strategies for gene therapy. Sci Amer. 276(6): 86–90.

Felgner PL, Gadek TR, Hom M, Roman R, Chun HU, Wenz M, Northrup JP, Ringold GM, and Danielsen M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Nat Acad Sci USA. 84: 7413–7417.

Felgner PL, Barenholz Y, Behr JP, Cheng SH, Cultis P, Huang L, Sessee JA, Seymour L, Szoka F, Thierry AR, Wagner E, and Wu G. 1997. Nomenclature for synthetic gene delivery systems. Hum Gene Ther. 8: 511–512.

Fernandez-Alonso M, Alvarez F, Estepa A, Blasco R, and Coll JM. 1999. A model to study fish DNA immersion vaccination by using the green fluorescent protein. J Fish Dis. 22: 237–241.

Fernandez-Alonso M, Rocha A, and Coll JM. 2001. DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. Vaccine. 19: 3067–3075.

Fraley R, Subramani S, Berg P, and Papahadjopoulos P. 1980. Introduction of liposome-encapsulated SV40 DNA into cells. J Biol Chem. 255: 10431–10435.

Fraley R, Straubinger RM, and Rule G. 1981. Liposome-mediated delivery of deoxyribonucleic acid to cells: Enhanced efficiency of delivery related to lipid composition and incubation conditions. Biochem. 20: 6978–6987.

Gao X and Huang L. 1995. Cationic liposome-mediated gene transfer. Gene Ther. 2: 710–722.

Garver KA, Conway CM, Elliott DG, and Kurath G. 2005. Analysis of DNA vaccinated fish reveals antigen in muscle, kidney and thymus, and transient hisopathological changes. Mar Biotech. 7: 540–553.

Garver KA, Conway CM, and Kurath G. 2006. Introduction of salmon translation stop codons into the viral glycoprotein gene in a fish DNA vaccine eliminates induction of protective immunity. Mar Biotech. 8: 351–356.

Gilmore RD, Engelking HM, Manning DS, and Leong JC. 1988. Expression in Escherichia coli of an

epitope of the glycoprotein of infectious hematopoietic necrosis virus protects against viral challenge. Nat Biotech. 6: 295–300.

Gomez-Chiarri M and Chiaverini LA. 1999. Evaluation of eukaryotic promoters for the construction of DNA vaccines for aquaculture. Genet Anal: Biomol Engin. 15: 121–124.

Gomez-Chiarri M, Livingston SK, Muro-Cacho C, Sanders S, and Levine RP. 1996. Introduction of foreign genes into the tissue of live fish by direct injection and particle bombardment. Dis Aquat Org. 27: 5–12.

Hansen E, Fernandes K, Goldspink G, Butterworth P, Umeda PK, and Chang KC. 1991. Strong expression of foreign genes following direct injection into fish muscle. FEBS Lett. 290: 73–76.

Harashima H, Shinohara Y, and Kiwada H. 2001. Intracellular control of gene trafficking using liposomes as drug carriers. Eur J Pharmaceut Sci. 13: 85–89.

Harbottle H, Plant KP, and Thune RL. 2005. DNA vaccination against channel catfish virus results in minimal immune response and is not efficacious against challenge. J Aquat Anim Health 17: 251–262.

Horne MT and Ellis AE. 1988. Strategies of fish vaccination. In: Fish vaccination (ed. Ellis AE). Academic Press, San Diego, CA. pp 55–66.

Jimenez J, Coll M, Salguero FJ, and Tafalla C. 2006. Co-injection of interleukin 89 with the glycoprotein from viral haemorrhagic septicaemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*). Vaccine. 24: 5615–5626.

Kanellos T, Sylvester ID, D'Mello F, Howard CR, Mackie A, Dixon PF, Chang KC, Ramstad A, and Midtlyng OJ. 2006. DNA vaccination can protect Cyprinis carpio against spring viraemia of carp virus. Vaccine. 24(23): 4927–4933.

Kanellos T, Sylvester ID, Ambali AO, Howard, CR, and Russell PH. 1999a. The safety and longevity of DNA vaccines for fish. Immunol. 96: 307–313.

Kanellos T, Sylvester ID, Howard CR, and Russell PH. 1999b. DNA is as effective as protein at inducing antibody in fish. Vaccine. 17: 965–972.

Kibenge FSB, Kibenge MJT, and McDougall J. 2003. In: Miller O and Cipriano RC (Tech. coords). International response to infectious salmon anemia prevention, control and eradication: Proceedings of a symposium, September 3–4, 2002, New Orleans, LA. Tech. Bull. 1902. Washington, DC, US Department of Agriculture, Animal and Plant Health Inspection Service; US Department of the Interior, US Geological Survey; US Department of Commerce, National Marine Fisheries Service 2003. pp 39–49.

Kibenge FSB, Kibenge MJT, McKenna PK, Stothard P, Marshall R, Cusack RR, and McGeachy S. 2001. Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. J Gen Virol. 82: 2869–2879.

Kibenge FS, Kibenge MJ, Groman D, and McGeachy S. 2006. In vivo correlates of infectious salmon anemia virus pathogenesis in fish. J Gen Virol. 87: 2645–2652.

Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, and Leong JA. 2000. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. J Virol. 74: 7048–7054.

Kumar SR, Ishaq Ahamed VP, Sarthi MM, Nazeer Basha A, and Sahul Hameed AS. 2008. Immunological responses of *Penaeus monodon* to DNA vaccine and its efficacy to protect shrimp against white spot syndrome virus (WSSV). Fish Shellfish Immunol. 24: 467–478.

Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, and La Patra SE. 2006. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine. 16: 345–354.

Kurath G. 2005. Overview of recent DNA vaccine development for fish. In: Progress in Fish Vaccinology (ed. Midlyng PJ) Karger, Basel. pp 201–213.

Kurath G. 2008. Biotechnology and DNA vaccines for aquatic animals. Rev Sci Tech, Off Int Epizoot. 27(1): 175–196.

Kurath G, Purcell MF, and Garver KA. 2007. Fish rhabdovirus models for understanding host response to DNA vaccines. CAB Reviews: Perspective in Agriculture, Veterinary Science, Nutrition and Natural Resources 2, No. 048. http://www. cababstractsplus.org/cabreviews (Online ISSN 1749–6848).

Kutzler MA and Weiner DB. 2008. DNA vaccines: ready for prime time? Nat Rev Genet. 9: 776–788.

LaPatra SE, Turner T, Lauda KA, Jones GR, and Walker S. 1993. Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. J Aquat Anim Health. 5: 165–71.

LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, and Kurath G. 2000. The dose-dependent effect on protection and humoral response to a DNA vaccine against infectious hematopoietic necrosis (IHN) virus in subyearling rainbow trout. J Aquat Anim Health. 12(3): 181–188.

LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, and Kurath G. 2001.

Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine. 19: 4011–4019.

Lee JY, Hirono I, and Aoki T. 2000. Stable expression of a foreign gene, delivered by gene gun, in the muscle of rainbow trout, *Oncorhynchus mykiss*. Mar Biotechnol. 2: 254–258.

Leitner WW. 2001. Myth, menace or medical blessing? The clinical potential and the problems of genetic vaccines. EMBO Reports. 2: 168–170.

Leong JC, Fryer JL, and Winton JR. 1988. Vaccination against infectious haematopoietic necrosis. In: Fish Vaccination (ed. Ellis AE). 1st ed. Academic Press, San Diego, CA. pp 193–203.

Leong JC, Anderson E, Bootland LM, Chiou P-W, Johnson M, Kim C, Mourich D, and Trobridge G. 1997. Fish vaccine antigens produced or delivered by recombinant DNA technologies. In: Fish Vaccinology (eds. Gudding R, Lillehaug A, Midtlyng PJ, and Brown F). Karger, Basel. Dev Biol Stand. 90: 267–277.

Leong JC, Crippen T, Drennan J, Johnson M, Jordan D, Kim CH, Simon B, and Thomann E. 2000. Development of DNA vaccines for fish. Suisan Zoshoku. 48: 285–290.

Levraud JP, Boudinot P, Collin F, Benmansour A, Peyrieras N, Herbommel P, and Lutfalla G. 2007. Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system. J Immunol. 178: 4385–4394.

Liu MA, Wahren B, and Hedestam GBK. 2006. DNA Vaccines: recent developments and future possibilities. Human Gene Ther. 17(11): 1051–1061.

Long S, Wilson M, Bengten E, Bryan L, Clem LW, Miller NW, and Chinchar VG. 2004. Identification of a cDNA encoding catfish interferon. Dev Comp Immunol. 28: 97–111.

Lorenzen N and Olesen NJ. 1997. Immunization with viral antigens: viral haemorrhagic septicaemia. Dev Biol Stand. 90: 201–209.

Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, and Davis HL. 1998. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) following DNA vaccination. Fish Shellfish Immunol. 8: 261–270.

Lorenzen N, and LaPatra SE. 1999. Immunity to rhabdoviruses in rainbow trout: the antibody response. Fish Shellfish Immunol. 9: 345–60.

Lorenzen E, Einer-Jensen K, Martinussen F, LaPatra SE, and Lorenzen N. 2000. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose–response and time course study. J Aquat Anim Health. 12: 167–180.

Lorenzen E, Lorenzen N, Einer-Jensen K, Brudeseth BE, and Evensen O. 2005. Time course study of an in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) fry. Fish Shellfish Immunol. 19: 27–41.

Lorenzen N and LaPatra SE. 2005. DNA vaccines for aquacultured fish. Rev. Scientif. et Technique—Off. Int Epizoot. 24: 201–213.

Lorenzen N, Lorenzen E, Einer-Jensen K, and La Patra SE. 2002a Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Dev Comp Immunol. 26: 173–179.

Lorenzen N, Lorenzen E, Einer-Jensen K, and La Patra SE. 2002b. DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. Fish Shellfish Immunol. 12: 439–453.

Lorenzen E, Einer-Jensen K, Rasmussen JS, Kjaer TE, Collet B, Secombes CJ, and Lorenzen N. 2009. The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. Vaccine. 27(29): 3870–3880.

Mahato RI, Rolland A, and Tomlinson E. 1997. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. Pharm Res. 14: 853–859.

McDougall J, Kibenge FSB, Evensen O. 2001. Vaccination against infectious salmon anemia virus (ISAV). In: Proceedings of the 10th International conference of the European Association of Fish PathologistsSeptember 9–14, 2001: Dublin (Trinity College) (ed. Hiney MH). Dublin, European Association of Fish Pathologists: P-192.

McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, and Ellis AE. 2003. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injections and duration of protection–early protection correlates with Mx expression. Fish Shellfish Immunol. 15: 39–50.

Mikalsen AB, Torgersen J, Alestrom P, Hellermann AL, Koppang EO, and Rimstad E. 2004. Protection of Atlantic salmon, *Salmo salar* against infectious pancreatic necrosis after DNA vaccination. Dis Aquat Org. 60: 11–20.

Mikalsen AB, Sindre H, Torgersen J, and Rimstad E. 2005. Protective effects of a DNA vaccine expression the infectious salmon anemia virus hemagglutinin-esterase in Atlantic salmon. Vaccine 23: 4895–4905.

Milev-Milovanovic I, Long S, Wilson M, Bengten E, Miller NW, and Chinchar VG. 2006. Identification

and expression analysis of interferon gamma genes in channel catfish. Immunogenet. 58: 70–80.

Moy R. 1999. Strid om genmanipulert laks—en vaksine kan skade salget. Aftenposten. September 8, 1999.

Nakhla AN, Szalai AJ, Banoub JH, Keough KMW. 1997. Serum anti-LPS antibody production by rainbow trout (*Oncorhynchus mykiss*) in response to the administration of free and liposomally-incorporated LPS from *Aeromonas salmonicida*. Fish Shellfish Immunol. 7: 387–401.

Nakhla AN, Szalai AJ, Bancub JH, and Keough KMW. 1994. Uptake and biodistribution of free and liposomally incorporated lipopolysaccharide of Aeromonas salmonicida administered via different routes to rainbow trout (*Oncorhynchus mykiss*). J Liposome Res. 4: 1029–1048.

Nusbaum KE, Smith BS, Delmocentes P, and Bird RC. 2002. Protective immunity induced by DNA vaccination of channel catfish with early and late transcripts of the channel catfish herpesvirus (IHV-1). Vet Immunol and Immunopath. 84: 151–168.

Oberg LA, Wirkkula J, Mourich D, and Leong JC. 1991. Bacterially expressed nucleoprotein of infectious hematopoietic necrosis virus augments protective immunity induced by the glycoprotein vaccine in fish. J Virol. 65(8): 4486–4489.

Pedroso de Lima MC, Simoes S, Pires P, Faneca H, and Duzgunes N. 2001. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. Adv Drug Deliv Rev. 47: 277–294.

Pisetsky DS. 1995. Immunologic consequences of nucleic acid therapy. Antisense Res Develop. 5: 219–225.

Pouton CW and Seymour LW. 2001. Key issues in non-viral gene delivery. Adv Drug Deliv Rev. 46: 187–203.

Power CA, Markham RJF, and Donald AW. 1990. Uptake and tissue distribution of liposomes after intraperitoneal administration to rainbow trout, *Oncorhynchus mykiss* (Richardson): a preliminary report. J Fish Dis. 13: 329–332.

Prasmickaite L. 2002. Photochemical Transfection for Site-Directed Gene Delivery—an In Vitro Study. Dissertation. Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital.

Purcell MK, Kurath G, Garver KA, Herwig RP, and Winton JR. 2004. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection of DNA vaccination. Fish Shellfish Immunol. 17: 447–462.

Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, and Park LK. 2006. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol. 43: 2089–2106.

Purcell MK, Laing KJ, Woodson JC, Thorgaard GH, and Hansen JD. 2009. Characterization of the interferon genes in homozygous rainbow trout reveals two novel genes, alternate splicing and differential regulation of duplicated genes. Fish Shellfish Immunol. 26: 203–304.

Rahman A and Maclean N. 1992. Fish transgene expression by direct injection into fish muscle. Mol Mar Biol Biotechnol. 1: 286–289.

Rajeshkumar S, Venkatesan C, Sarathi M, Sarathbabu V, Thomas J, Anver Basha K, and Sahul Hameed AS. 2009. Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from whitespot syndrome virus (WSSV). Fish Shellfish Immunol. 26: 429–437.

Ramstad A, Romstad AB, Knappskog DH, and Midtlyng PJ. 2007. Field validation of experimental challenge models for IPN vaccines. J Fish Dis. 30(12): 723–731.

Robertsen R, Bergan V, Rokenes T, Larsen R, and Albuquerque A, 2003. Atlantic salmon interferon genes: cloning, sequence analysis, expression and biological activity. J Interferon Cytokine Res. 10: 601–612.

Romøren K, Thu BJ, Smistad G, and Evensen Ø. 2002. Immersion delivery of plasmid DNA I. A study of the potentials of a liposomal delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. J Control Release. 85: 203–213.

Romøren K, Thu BJ, and Evensen Ø. 2004. Expression of luciferase in selected organs following delivery of naked and formulated DNA to rainbow trout (*Oncorhynchus mykiss*) by different routes of administration. Fish Shellfish Immunol. 16(2): 251–264.

Ruiz S, Tafalla C, Cuesta A, Estepa A, and Coll JM. 2008. In vitro search for alternative promoters to the human immediate early cytomegalovirus (IE-CMV) to express the G gene of viral haemorrhagic septicaemia virus (VHSV) in fish epithelial cells. Vaccine. 26: 6620–6629.

Rucker RR, Whipple WJ, Parvin JR, and Evans CA. 1953. A contagious disease of sockeye salmon possibly of virus origin. USFWS Fisheries Bull. 54: 35–46.

Russell PH, Kaknellos T, Sylvester ID, Chang KC, and Howard CR. 1998. Nucleic acid immunisation with a

reporter gene results in antibody production in goldfish (*Carassius auratus* L.). Fish Shellfish Immunol. 8: 121–128.

Samson SL, Paramchuk WJ, and Gedamu L. 2001. The rainbow trout metallothionein-B gene promoter: contributions of distal promoter elements to metal and oxidant regulation. Biochim Biophys Acta. 1517: 202–211.

Sanchez E, Coll JM, and Tafalla C. 2007. Expression of inducible CC chemokines in rainbow trout (*Oncorhynchus mykiss*) in response to a viral haemorrhagic septicaemia virus (VHSV) DNA vaccine and interleukin 8. Dev Comp Immunol. 31: 916–926.

Schaperclaus W. 1938. Die Schadigungen dere deutschen Fischerei deurch Fischparastein und Fischkrankheiten. Allegemeine Fischerei-Zeitung. 41: 256–259, 267–270.

Secombes C. 2008. Will advances in fish immunology change vaccination strategies? Fish Shellfish Immunol. 25: 409–416.

Seo JI, Kim KH, Oh MJ, Nam SW, Kim YT, and Choi TJ. 2006. Protection of flounder against hirame rhabdovirus (HIRRV) with a DNA vaccine containing the glycoprotein gene. Vaccine. 24: 1009–1015.

Simoes S, Stepushkin V, Pretzer E, Dazin P, Gaspar R, Pedroso de Lima MC, and Duzgunes N. 1999. Transfection of human macrophages by lipoplexes via the combined use of transferrin and pH-sensitive peptides. J Leukoc Biol. 65: 270–279.

Smail DA. 1999. Viral haemorrhagic septicaemia. In: Fish Diseases and Disorders: Viral, Bacterial, and Fungal Infections (eds. Woo PTK and Bruno DW). CAB International. pp 123–147.

Smith J, Zhang Y, and Niven R. 1997. Toward development of a non-viral gene therapeutic. Adv Drug Deliv Rev. 26: 135–150.

Somia N and Verma IM. 2000. Gene therapy: trials and tribulations. Nat Rev Genet. 1: 91–99.

Sommerset I, Lorenzen E, Lorenzen N, Bleie H, and Nerland A. 2003. DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot. Vaccine. 21: 4661–4667.

Sommerset I, Skern R, Biering E, Bleie H, Fiksdal I, Grove S, and Nerland A. 2005. Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. Fish Shellfish Immunol. 18: 13–29.

Stolte EH, Savelkoul HFJ, Wiegertjes G, Flik G, and Werburg-van Kemenade LBM. 2008. Differential expression of two interferon gamma genes in common carp (*Cyprinus carpio* L.). Dev Comp Immunol. 32: 1467–1481.

Sudha PM, Low S, Kwang J, and Gong Z. 2001. Multiple tissue transformation in adult zebrafish by gene gun bombardment and muscular injection of naked DNA. Mar Biotechnol. 3: 119–125.

Szoka FC Jr, Xu Y, and Zelphati O. 1996. How are nucleic acids released in cells from cationic lipid-nucleic acid complexes? J Liposome Res. 6: 567–587.

Tafalla C, Chico V, Perez L, Coll JM, and Estepa A. 2007. In vitro and in vivo differential expression of rainbow trout (*Oncorhynchus mykiss*) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. Fish Shellfish Immunol. 23: 210–221.

Takano C, Iwahori A, Hirono I, and Aoki T. 2004. Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after DNA vaccination. Fish Shellfish Immunol. 17: 367–374.

The European Agency for the Evaluation of Medicinal Products, Committee for veterinary medicinal products. 1998. Note for guidance: DNA vaccines non-amplifiable in eukaryotic cells for veterinary use. CVMP/IWP/07/98-FINAL. pp 1–7.

The European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products. 2001. Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products. CPMP/BWP/3088/99. pp 1–31.

Thierry AR and Sullivan SM. 1997. Optimization of lipoplex formulations for intravenous gene delivery. J Liposome Res. 7: 143–223.

Thierry R, Cozien J, Cabon J, Lamour F, Baud M, and Schneemann A. 2006. Induction of a protective immune response against viral nervous necrosis in the European Sea Bass *Dicentrarchus labrax* by using betanodavirus-like particles. J Virol. 80: 10201–10207.

Tian J-Y, Sun X-!, and Chen X-G. 2008. Formation and oral administration of alginate microspheres loaded with pDNA coding for lymphocystis disease virus (LCDV) to Japanese flounder. Fish Shellfish Immunol. 24: 592–599.

Tonheim TC, Bøgwald J, and Dalmo RA. 2008. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol. 25(1-2): 1–18.

Tonheim TC, Leirvik J, Løvoll M, Myhr AI, Bøgwald J, and Dalmo RA. 2007. Detection of supercoiled

plasmid DNA and luciferase expression in Atlantic salmon (*Salmo salar* L.) 535 days after injection. Fish Shellfish Immunol. 23(4): 867–876.

Torgersen J, Collas P, and Alestrom P. 2000. Gene-gun-mediated transfer of reporter genes to somatic zebrafish (*Danio rerio*) tissues. Mar Biotechnol. 2: 293–300.

Traxler GS, Anderson E, LaPatra SE, Richard J, Shewmaker B, and Kurath G. 1999. Naked DNA vaccination of Atlantic salmon Salmo Salar against IHNV. Dis Aquat Organ. 30(3): 183–190.

Tucker C, Endo M, Hirono I, and Aoki T. 2001. Assessment of DNA vaccine potential in juvenile Japanese flounder *Paralichthys olivaceus*, through the introduction of reporter genes by particle bombardment and histopathology. Vaccine. 19: 801–809.

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, J, Gromkowski SH, Deck RR, Dewitt CM, Friedman A, Hawe IA, Leander KR, Martinez D, Perry HC, Shiver JW, Montgomery DI, and Liu MA. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. 259: 1745–1749.

Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Köllner B, Dalmo RA, Vesely T, Ototake M, and Fischer U. 2008. Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. Dev Comp Immunol. 32: 239–252.

Van de Wetering P. 1999. Gene delivery with cationic polymers. Synthesis, characterization and biological evaluation of poly(2-(dimethylamino)ethyl methacrylate) and related polymers. Dissertation. Utrecht University, Faculty of Pharmacy.

Walter E, Thiele L, and Merkle HP. 2001. Gene delivery systems to phagocytic antigen-presenting cells. STP Pharma Sci. 11: 45–56.

Webster RG and Robinson HL. 1997. DNA vaccines: a review of developments. Biodrugs. 8: 273–292.

Wiethoff CM and Middaugh CR. 2003. Barriers to nonviral gene delivery. J Pharm Sci. 92: 203–217.

Winton JR. 1997. Immunization with viral antigens: infectious hematopoietic necrosis. In: Fish Vaccinology (eds. Gudding R, Lillehaug A, Midtlyng PJ, and Brown F). Karger, Basel. Dev Biol Stand. 90: 211–220.

Wolff JA, Malone RW, Williams P, Chong W, Ac-Sadi G, Jani A, and Felgner PL. 1990. Direct gene transfer into mouse muscle in vivo. Science. 247: 1465–1468.

Yasuike M, Kondo H, Hirono I, and Aoki T. 2007. Difference in Japanese flounder, *Paralichthys olivaceus* gene expression profile following hirame rhabdovirus (HIRRV) G and N protein DNA vaccination. Fish Shellfish Immunol. 23: 531–541.

Zallen DT. 2000. US gene therapy in crisis. Trends Genet. 16: 272–275.

Zheng FR, Sun XO, Liu HZ, and Zhang JX. 2006. Study on the distribution and expression of a DNA vaccine against lymphocystis disease virus in Japanese flounder (*Paralichthys olivaceus*). Aquaculture 261: 1128–1134.

Zhdanov RI, Podobed OV, and Vlassov VV. 2002. Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. Bioelectrochem. 58: 53–64.

Zou J, Yasutoshi YR, Dijkstra JM, Sakai M, Ototake M, and Secombes C. 2004. Identification of an interferon gamma homologue in fugu, *Takifugu rubripes*. Fish Shellfish Immunol. 17: 403–408.

Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, Secombes C. 2005. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. J Immunol. 175: 2484–2494.

Zou J, Tafalla C, Truckle J, and Secombes C. 2007. Identification of a second group of type 1 IFNs in fish sheds light on IFN evolution non vertebrates. J Immunol. 179: 3859–3871.

Zuidam NJ and Barenholz Y. 1998. Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. Biochim Biophys Acta. 1368: 115–128.

Part 5
Embryogenesis and Stem Cells

Chapter 11

Egg Transcriptome, the Maternal Legacy to the Embryo

Juan Martin Traverso, Alexis Fostier, and Julien Bobe

INTRODUCTION

Fish egg quality can be defined as the ability of the egg to be fertilized and subsequently develop into a normal embryo (Bobe and Labbe 2010). The unfertilized egg is an oocyte arrested in metaphase of the second meiotic division. This metaphase 2 oocyte is the final product of the oogenetic process that occurred within the ovary throughout oogenesis. The coordinated assembly of the egg is thus a long-time process during which several types of molecules accumulate within the oocyte (Tata 1986). Among those molecules, maternal mRNAs (or maternally inherited mRNAs) accumulate in the oocyte throughout oogenesis. After fertilization, maternal factors support early embryonic development until activation of zygotic transcription. The initiation of zygotic transcription occurs during the "maternal-embryo transition" (MET). In fish, as in other lower vertebrates, MET occurs at the mid-blastula stage and is also known as "mid-blastula transition" (MBT) (Kane and Kimmel 1993). During the first steps of embryonic development, the embryo exhibits rapid and synchronous cleavage. In zebrafish, MBT occurs at cycle 10 and is characterized, in addition to the initiation of zygotic transcription, by the increase of cell cycle length, the loss of cell synchrony, and the appearance of motility (Kane and Kimmel 1993). The activation of zygotic transcription is paralleled by the differential degradation of maternally inherited mRNAs (Mathavan et al. 2005; Giraldez et al. 2006). The switch from maternal to zygotic gene expression is, however, more progressive than originally thought (Fig. 11.1). Not all maternal mRNAs are degraded at MBT and many maternal mRNAs continue to influence embryo development at later stages (Pelegri 2003; Wagner et al. 2004). Similarly, some recently identified genes are transcribed in the zebrafish embryo prior to MBT (Mathavan et al. 2005).

Over the past decades, the role of maternal mRNAs in early development has received increasing attention and has been investigated in mammals and in several model species. In fish, the participation of maternal mRNAs in the control or regulation of egg quality has been hypothesized (Brooks et al. 1997). Yet, our knowledge of the maternal mRNAs present in the fish oocyte remains limited. Similarly, the links between maternal mRNAs and oocyte quality are poorly understood despite the recent use of transcriptomic tools to address this

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

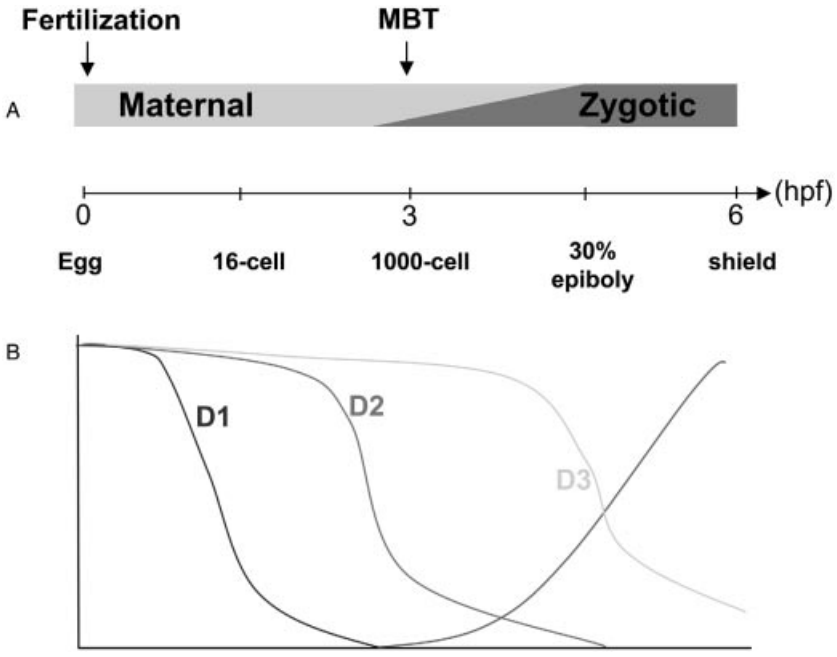


Figure 11.1. (**A**) Schematic representation of maternal and zygotic gene activities during zebrafish embryonic development. Mid-blastula transition (MBT) is indicated. (**B**) Degradation patterns of maternal mRNAs throughout embryonic development. Slow (D1), medium (D2), and fast (D3) degrading patterns are shown. (For color detail, please see the color plate section.)

question. In the present chapter, we review available information on the maternal mRNAs present in fish egg and early embryo with special attention for the molecular functions that could participate in the regulation or control of the oocyte ability to develop after fertilization. Data obtained in several fish species using DNA microarrays, serial analysis of gene expression (SAGE), and real-time quantitative reverse transcription–polymerase chain reaction (QPCR) are reviewed. Whenever possible, functional information generated by genetic screens and knock-down studies is also included. The contribution of maternal mRNAs to specific stages and events of embryonic development are also discussed and several examples are given. The overall goal of this chapter is to provide an overview of the maternal mRNAs present in the fish egg as well as any information on the functional roles of specific maternal mRNAs in early development.

MOLECULAR PORTRAIT OF THE FISH OOCYTE

Transcriptomic Analysis of the Oocyte and Early Embryo

In the past few years, egg and early embryo transcriptome have been investigated using newly available genomic tools in several fish species. A first study was performed in 2003 (Lo et al. 2003) in zebrafish (*Danio rerio*) using an approximately 3000 gene (3K) cDNA microarray to analyze several embryonic stages, including cleavage stage (Kimmel et al. 1995), the unfertilized egg stage being used as methodological reference. However, this study was focused on later embryonic events (i.e., myogenesis), and very little information on the nature of maternal mRNA was provided. A zebrafish 16K oligonucleotide (oligo) microarray was used to analyze mRNA expression throughout embryonic

development (Mathavan et al. 2005). A subset of 622 genes displaying maximum abundance at the unfertilized egg stage was identified. This set of genes was further separated in three groups based on the pattern of mRNA degradation during early development. A first group of 221 genes was characterized by a rapid degradation, prior to MBT. In addition to a large number of previously uncharacterized genes, this subgroup contained zona pellucida genes as well as members of the lectin family. A second group of 259 genes persisted until MBT and were slowly degraded by the end of the blastula stage. This group contained genes involved in cell cycle (e.g., cyclin b1), cell death/apotosis, cell growth, as well as chaperone proteins and nucleoskeletal proteins of the nuclear membrane during cell division. Finally, a third group of 142 genes underwent very slow transcript degradation throughout early embryogenesis. These patterns of RNA degradation during early development (Fig. 11.1) were further confirmed by other investigators (Ferg et al. 2007). The presence of specific maternal mRNAs, such as zona pellucida genes and lectins, was also confirmed in zebrafish by other investigators using a combination of microarray analysis of ovarian tissue and RNA blot hybridization (Wen et al. 2005). More recently, 1419 expressed sequence tags from Atlantic halibut (*Hippoglossus hippoglossus*) 2-cell embryos were generated and analyzed (Bai et al. 2007). Almost 800 different genes were deduced from ESTs. One of the main findings of the study was that maternal mRNA in the 2-cell embryo contained a high proportion of mitochondrial transcripts. In contrast, only 2 ESTs coding for ribosomal proteins were identified. Among non-mitochondrial gene, maternal mRNAs for cyclin b1, cathepsin L, and histone proteins H1m, H2a, and H3 were reported. A great deal of information was also generated by SAGE analysis of zebrafish fully grown ovarian follicles (Knoll-Gellida et al. 2006). The three most expressed transcripts in ovarian follicles were a rhamnose-binding lectin, beta-actin 2, and a transcribed locus similar to the H2b histone family. Recently, the transcriptome of rainbow trout (*Oncorhynchus mykiss*) unfertilized eggs after natural, hormonally induced, and photoperiod-controlled ovulation was studied using a 9K cDNA microarray (Bonnet et al. 2007b). The analysis showed that the levels of 26 maternal mRNAs were modified after hormonally induced and photoperiod-controlled ovulation. The overexpression of apoliprotein C1 (*apoc1*) and tyrosine–protein kinase HCK mRNAs in unfertilized eggs after photoperiod- or hormone-induced ovulation was further confirmed by QPCR. This study showed that external factors applied during oogenesis could trigger changes in the abundance of maternal mRNAs in the unfertilized egg. This suggests that the effects of external factors on fish egg quality, and subsequent developmental capacities, could be mediated, at least in part, by changes in the levels of maternal mRNAs. (Throughout this chapter, gene/mRNA names will be italicized and protein names will not be italicized but will have their first letter capitalized in agreement with official zebrafish gene/protein nomenclature.)

Messenger RNA Present in the Oocyte and Early Embryo Before the Mid-Blastula Transition

As in other animals, maternal factors have been shown to be involved in both somatic and germ cell lineages fate during fish embryogenesis. For instance, yolk depletion, yolk grafting, and RNase injection experiments have shown that a mesoderm- and endoderm-inducing signal reside in the yolk (Mizuno et al. 1999; Ober and Schulte-Merker 1999; Chen and Kimelman 2000). Besides, the ablation of cytoplasm at the distal ends of the first and second cleavage furrows resulted in a severe reduction in the number of germ cells (Hashimoto et al. 2004). Further experiments have been developed to identify mRNA among these maternal factors.

Zebrafish has been used intensively as a powerful model system for investigation of vertebrate development because of its low rearing cost, high fecundity, 3-month generation time, accessibility of the embryo, optical transparency of the embryo, and availability of mutants (Schier and Talbot 2005). However, other laboratory models, such as medaka (*Oryzias latipes*) in which many inbred strains are available, have also been used. Less frequently, farmed fish have also been investigated.

Maternal origin of mRNA is recognized by their occurrence in the mature oocyte and/or embryo during pre-MBT development. However, the fully mature oocyte or ovulated oocyte might include some nondegraded remnant mRNA involved in oogenesis

and ovulation but not in early embryogenesis. Besides, an accurate knowledge of MBT timing is required, which is not always the case for all species. In zebrafish, MBT begins at the 512-cell stage, i.e., 2.75 hours postfertilization (hpf) at 28.5°C (Kimmel et al. 1995). In medaka, MBT in terms of first transcription of paternal genes in the life of the embryo has been studied using the detection of gene polymorphisms between male and female belonging to two inbred strains (Aizawa et al. 2003). In medaka, MBT begins a little later than in zebrafish at the late blastula stage, i.e., 8.25 hpf at 26°C (Iwamatsu 2004). However, both decrease and/or exhaustion of maternal products might be detected at the onset of the MBT showing the complexity of the MBT process.

Various methods have been used to detect maternal mRNAs such as Northern blot, semiquantitative RT-PCR, real-time QPCR, in situ hybridization, cDNA, or oligonucleotide microarray hybridization; each of them having their own characteristics in terms of sensitivity and specificity. The specific mRNAs for which maternal inheritance has been investigated in fish often originated from the extensive studies developed in *Drosophila*. In a systematic determination of expression patterns of 3370 genes (about 25% of the genome) during *Drosophila* early embryogenesis, 53.7% were found exclusively maternally expressed and 11.5% showed both maternal and zygotic expression (Lecuyer et al. 2007). However, some of these maternally expressed genes are housekeeping genes or basic genes involved in the metabolism of the first cells but attention was paid mostly to particular genes able to determine the specific fate of an embryo. Thus, studied mRNAs were also selected because of their known function in zygotic embryo or in adult, and functional analyses are needed to determine their actual roles as maternal mRNAs in fish. Conclusions may be hard to draw because of the difficulties in designing experiments that control either a maternal mRNA or its zygotic counterpart independently. This is the case for the genes belonging to the nodal family of signaling molecules such as squint (*sqt*) involved in the early establishment of the embryonic axes. RNA interference might be another powerful method for determining the function of maternal transcripts during early embryonic development and has been used successfully in mammals (Nganvongpanit et al. 2006). This posttranscriptional gene silencing method is now based on the injection of a small double-stranded RNA containing one strand, which is complementary to the mRNA of the suppressed gene and catalyzing its targeted cleavage (Schyth 2008). However, in zebrafish, processed microRNAs (miRNAs) belonging to the miR-430 family have been shown to promote deadenylation and degradation of maternal mRNAs during early embryogenesis (Giraldez et al. 2006); short interfering RNA (siRNA) may cause a significant reduction in the endogenous levels of processed miR-430 and other miRNAs, leading to unspecific developmental defects (Zhao et al. 2008).

Another experimental opportunity to analyze the role of maternal mRNA is the use of natural or induced mutants with morphogenic defects during embryogenesis. The comparison between embryos issued from either a wild female or a female bearing a homozygous mutation involved in maternal mRNA synthesis helps to identify the specific role of maternal versus zygotic mRNAs (Dosch et al. 2004).

As in other species, fish germ cells form in a specialized germ plasm, which contains maternal mRNAs with critical functions in germ-cell specification, migration, and development (Hashimoto et al. 2004). Germ plasm is defined as a substance present in the cytoplasm of gametes, which is inherited by a few cells and determines the fate of the primordial germ cells (PGCs; Eddy 1975). The germ plasm is incorporated into a few embryonic cells, which subsequently give rise to PGCs, the first generation of the germ cell lineage. Several of these germ cells-linked mRNAs have been found in fish, mainly in the zebrafish, and subjected to detailed studies. This is the case for *vasa* (*vas*) encoding a member of the DEAD protein family (a large family of ATP-dependent RNA helicases that share a common protein motif with the single letter amino acid sequence D-E-A-D, i.e., Asp-Glu-Ala-Asp) (Knaut et al. 2000, 2002), *nanos1 homolog* (*nan*) encoding an RNA binding zinc finger protein (Koprunner et al. 2001), dead end (*dnd*) encoding an RNA-binding protein (Weidinger et al. 2003), the linker histone-like gene *h1m* (Muller et al. 2002; Wibrand

and Olsen 2002), *dazl* encoding a RNA-binding protein belonging to the deleted in azoospermia family (Hashimoto et al. 2004; Xu et al. 2007), *bruno*-like (*brul*) encoding an Elav-type RNA-binding protein showing three RNA-recognition motifs (Suzuki et al. 2000; Hashimoto et al. 2004), *askopos* (*kop*, meaning "without purpose" in Greek) encoding a nuclear protein (Blaser et al. 2005), *staufen1* and *staufen2* (*stau1* and *stau2*), encoding proteins with 5 RNA-binding domains and 1 tubulin-binding domain (Ramasamy et al. 2006), *germ cell less* (*gcl*) known in *Drosophila* to be a component of the germ plasma and required for PGCs formation (Scholz et al. 2004; Li et al. 2006), and *granulito* (*gra*) and a tudor domain-containing gene (*tdr7*) both expressed in germ cells granules (Strasser et al. 2008). Maternal mRNAs of germline-specific genes are localized to the distal ends of the first and second cleavage planes. However, the localization patterns of some of these RNAs indicate a compartmentalization of germ plasm with distinct RNA composition along the cleavage furrow (Theusch et al. 2006). Besides, germ cells-linked mRNA such as vasa may be preferentially stabilized in germ cells versus somatic cells in which they are degraded (Wolke et al. 2002).

However, most of the maternal mRNAs that have been identified were involved in the somatic fate. They may be involved early (e.g., E-cadherin) in epiboly, the spreading of the blastoderm over the yolk, which is the first morphogenetic movement of the teleost embryo (Kane et al. 2005). Then, following Peligri (Pelegri 2003), most of the maternal mRNAs can be classified according to their role in the establishment of dorsoventral (DV) polarity, for which three major signaling pathways have been implicated in the early zebrafish embryo (Wnt/catenin, Wnt/calcium, and bone morphogenetic protein signaling), in the anterior–posterior axis determination, and in the germ layer specification (Pelegri 2003). Besides, they can be involved in the mobilization of yolk proteins during embryogenesis (e.g., cathepsins D and L (Kwon et al. 2001). Finally, maternal factors are involved in embryo energy metabolism (Mendelsohn and Gitlin 2008). This might be the case of the abundant maternal mRNA originating from mitochondrial genes of Atlantic halibut (Bai et al. 2007).

ROLE OF MATERNAL mRNAS IN OOCYTE QUALITY AND EARLY EMBRYONIC DEVELOPMENT

Insight from Mutational and Gene Knock-Down Studies

Early embryonic development is driven by maternal factors, which are produced during oogenesis and stored in the oocyte's cytoplasm as mRNAs, proteins, and other biomolecules (Tata 1986). As already indicated, these maternally deposited gene products act in the control of early embryonic cleavage stages and the establishment of the different body axes. Moreover, the maternal influence on embryogenesis persists beyond the onset of zygotic transcription at the MBT, which is evidenced by the persistence and activity of some maternal transcripts after the MBT, functioning together with zygotic gene products (Wagner et al. 2004). The maternal factors in fish oogenesis and their contribution to early embryonic development have been thoroughly reviewed (Pelegri 2003). In this section, we focus on the most recent data obtained on maternal factors affecting early embryonic development.

Gene Knock-Down Studies for Maternal Factors Involved in Germline Establishment

The work described below has been done in a great deal using antisense morpholino oligonucleotides (MOs) microinjection (Table 11.1). MOs are synthetic short chains of about 25 subunits, composed of a nucleic acid base, like in DNA and RNA oligonucleotides, except that they have a morpholine ring rather than a ribose ring. This feature allows MOs to retain Watson-Crick base pairing properties, while rendering them resistant to nucleases, and therefore particularly stable. Antisense MOs are thus designed to target specific mRNAs and perform gene knockdown acting via a steric blocking mechanism. A recent and complete review on MOs-mediated knockdown, considering advantages and disadvantages of this method, can be found in Eisen and Smith (Eisen and Smith 2008).

In zebrafish, the *vasa* RNA rather than the protein is initially localized to the germ plasm (Knaut et al. 2000; Raz 2000) and the microinjection of a vasa

Table 11.1. Role of Maternal mRNAs in Oocyte Quality and Early Embryonic Development: Recent Findings from Mutational and Gene Knock-Down Studies.

Maternal mRNA	Outcome (O)/ Phenotype (P)	Fish Species	Technique	References
Dead end	P: impaired migration and survival of PGCs	Zebrafish	Gene knock-down	Weidinger et al. 2003
stau1/stau2			Gene knock-down	Ramasamy et al. 2006
tdr7	P: disturbance of germ cell granules number and morphology	Zebrafish	Transgenesis	Strasser et al. 2008
kop	P: no effect on PGCs development	Zebrafish	Transgenesis	Blaser et al. 2005
β-catenin-2	P: dorsal and anterior defects, ventralization	Zebrafish	Gene knock-down	Bellipanni et al. 2006
nkd2a	P: inhibition of Wnt signaling pathway	Zebrafish	Protein overexpression	van Raay et al. 2007
sqt	P: perturbation of dorsoventral axis formation (after MBT)	Zebrafish	Mutant crossing, drug treatment, protein overexpression	Hagos et al. 2007
	O: degree of *sqt*-related perturbations influenced by genetic background and environment		Gene knock-down	Pei et al. 2007b
foxh1	P: head and dorsal axis formation abnormalities	Zebrafish	Gene knock-down	Pei et al. 2007a
smad2/3	P: mesendodermal defects	Zebrafish	Mutant fish generation	Jia et al. 2008
chordin^{UT006}	P: ventralization of body axis, defects in yolk sac blood vessels, laterality defects in internal organs, bone malformations	Medaka	Gene knock-down	Takashima et al. 2007
shh	O: involvement of carp *shh* in pattern formation?	Common carp	RT PCR/RACE	Wang et al. 2007
irf6	P: epiboly arrest, rupture of the embryo at late gastrula stage	Zebrafish	Gene knock-down, dominant negative protein expression	Sabel et al. 2009
pou5f1/oct4	P: severe delay in epiboly progression	Zebrafish	Mutant embryos	Lachnit et al. 2008
brg1	P: defects in formation of craniofacial structures	Zebrafish	Gene knock-down, mutant crossing	Eroglu et al. 2006
mys	P: defects at the somite boundary formation	Zebrafish	Gene knock-down	Kotani and Kawakami 2008

antisense morpholino oligonucleotide did not disturb the germline (Braat et al. 2001). The respective contribution of vasa mRNA and protein in germ cell development remain unclear. While it is possible that in zebrafish it is the vasa RNA and not the protein that is important for the early determination of germ plasm (Knaut et al. 2000), a modulation of Vasa activity by posttranslational modification has also been suggested (Raz 2000). As well, it is noteworthy that in zebrafish *vasa* RNA expressed ectopically during embryogenesis is unable by itself to alter the number or position of the PGCs (Weidinger et al. 1999). Furthermore, the analysis of the expression pattern of the medaka *vasa* homolog showed a uniform expression of the RNA until gastrulation, and a germ-cell specific expression only from this moment (Shinomiya et al. 2000). Data collected in several animal species (reviewed in Raz 2000) allows hypothesizing that the function of Vasa would be to preserve totipotency, i.e., by inhibiting expression of genes that would lead to somatic differentiation. Knock-down experiments have shown that Nanos (Koprunner et al. 2001), Dead End (Weidinger et al. 2003), Stau1 or Stau2 (Ramasamy et al. 2006) were necessary to ensure a proper migration and survival of germ cells. Besides, phenotypes were rescued by coinjection of *Drosophila* stau mRNA. Controversially, no effects on PGC specification, division, or migration upon inhibition of tdr7 translation were observed, but germ cell granule morphology and number were disturbed (Strasser et al. 2008). Finally, translation inhibition of *kop* RNA had no effect on PGCs development (Blaser et al. 2005).

Maternal Factors Involved in Pattern Formation

Wingless (Wnt) signaling controls a wide range of developmental processes, through two major pathways: the β-catenin-dependent or canonical pathway, and the β-catenin-independent or noncanonical one (reviewed in Widelitz 2005; Barker 2008; Liu et al. 2008, among many others). Recent work has shed light on the roles played by β-catenins in the formation of dorsal structures and neurectoderm. Bellipanni and coworkers have discovered a new β-catenin gene (β-*catenin-2*) and used targeted gene knockdown by microinjection of MOs to establish that maternal expression of this isoform is solely responsible for the formation of the dorsal organizer in the zebrafish (Bellipanni et al. 2006). As well, evidence was presented indicating that embryos showing the *ichabod* phenotype (a recessive maternal effect mutation, that presents severe dorsal and anterior defects during early development) lack maternal (but not zygotic) expression of β-*catenin-2*. Moreover, as MO β-*catenin-2* loss-of-function phenotypes were clearly the same as the ventralized *ichabod* phenotypes, it was suggested that the *ichabod* mutation is a regulatory mutation affecting the maternal expression of β-*catenin-2*.

Zebrafish β-catenins redundantly inhibited neurectoderm. Indeed, reduction of β-*catenin-2* expression alone resulted in ventralization, not dorsalization, and reduction of β-*catenin*-1 expression alone had no effect on DV or anteroposterior (AP) patterning (Bellipanni et al. 2006). However, when expression of both β-catenins was inhibited, a strong formation of neurectodermal tissue was observed, expressing posterior neural markers only occasionally seen in the tail-like appendage of *ichabod* embryos. These results add to the array of data indicating that AP patterning of the neurectoderm can take place in embryos lacking organizer tissue, as has also been shown in *Xenopus* (Reversade et al. 2005).

On the other hand, van Raay and coworkers recently identified zebrafish homologs of Naked Cuticle (Nkd), Nkd1, and Nkd2, which had previously been shown to inhibit the canonical Wnt signaling pathway. Zebrafish *nkd1* and *nkd2* exhibited different expression patterns, with *nkd1* showing a predominantly zygotic expression pattern, notably increasing after the MBT and in regions recapitulating active canonical Wnt signaling. In contrast, *nkd2* was found to be maternally and ubiquitously expressed (van Raay et al. 2007). Moreover, injection of *nkd1* or *nkd2a* mRNAs (and thus Nkd1 and Nkd2a protein overexpression) suppressed canonical Wnt signaling at various stages of early zebrafish development and also exacerbated defects in the noncanonical Wnt/PCP mutant *silberblick* (*slb/wnt11*), indicating that zebrafish Nkd1 and Nkd2 function as inhibitors of both canonical and noncanonical Wnt signaling.

The transforming growth factor beta (TGF-β) superfamily of secreted cytokines plays a key role in

embryogenesis signaling. Consequently, new data outlined below involves many members of this super family. Recent work on Squint (Sqt), a Nodal-related protein, re-evaluated the need of the maternal *sqt* transcript for embryogenesis in zebrafish, and a general requirement for this maternal transcript was ruled out by two different studies. The work of Hagos and colleagues analyzed embryos lacking both maternal and zygotic Sqt function, and revealed a variable requirement (depending on the genetic background of the mother) for maternal *squint* for the formation of dorsal and anterior tissues (Hagos et al. 2007). In these cases, however, maternal *sqt* played its role after the MBT. Nevertheless, it has been suggested that the dorsally localized maternal *sqt* mRNA could provide a necessary extra dose of Nodal signal because the dorsal side of the embryo needs Nodal function more than the other regions of the embryo (Kimelman 2006). As well, while genetic analysis demonstrated a requirement for maternal *sqt*, it did not support a role for maternal *sqt* during the cleavage stages for axis determination (Hagos et al. 2007). These data contrasted with the early role played by these signals during *Xenopus* development (Scharf and Gerhart 1983; Black and Gerhart 1986), but reconciled previous contradictory results in the published literature on the role played by maternal TGF-β signals in zebrafish embryogenesis (Aoki et al. 2002; Gore et al. 2005; Sun et al. 2006; Kotani and Kawakami 2008). In the other study, Pei and coworkers found that both maternal and zygotic Sqt was generally dispensable, and that *sqt* penetrance was influenced by genetic modifiers, by environmental temperature prior to gastrulation, by levels of residual Activin-like signaling levels, and by heat-shock protein 90 activity (Pei et al. 2007b). This same group also used splice-disrupting and translation-blocking MOs to reassess the role of the zebrafish Forkhead Box H1 (FoxH1) protein, which is a cotranscription factor recruited in response to the Nodal, Activin, and Vg1-secreted ligands of the TGF-β superfamily (Whitman 2001; Shi and Massague 2003). They found that a splice-disrupting MO yielded a phenotype consistent with previously reported *sur* mutants (Pogoda et al. 2000; Kofron et al. 2004), while blocking translation of the *foxh1* message with either of two nonoverlapping MOs yielded an earlier and much more severe phenotype, thus suggesting a critical role for maternal FoxH1, which in theory is uniquely targeted by the translation-blocking MOs (Pei et al. 2007a).

The transcription factors Snao 2 and Smad3 are intracellular effectors of Nodal signals (Kumar et al. 2001; Whitman 2001). Jia and colleagues generated specific dominant-negative alleles of *smad2*, *smad3a*, and *smad3b*, which they used to demonstrate that Smad2/3 activities were required for mesendoderm induction in zebrafish and that Nodal signals exerted their biological effects during embryonic development depending on Smad2/3 activities (Jia et al. 2008).

New insight on DV patterning has been provided by recent work on the Japanese killifish, *O. latipes*. Indeed, a novel *chordin* mutant (*chordin*^{UT006}) has recently been isolated and characterized in this species (Takashima et al. 2007), showing ventralization of the body axis, malformation of axial bones, over-bifurcation of yolk sac blood vessels, and laterality defects in internal organs. While chordin is a well-conserved gene required for early DV patterning of vertebrate embryos, medaka *chordin*^{UT006} embryos often exhibited a very severe phenotype, without head and trunk structures, suggesting that in this species, the dependency on chordin for DV patterning is higher than in other vertebrates.

Finally, we will mention the discovery of a maternal expression of the well-known morphogen *Sonic hedgehog* (*shh*) in the common carp *Cyprinus carpio*. This result contrasts with *shh* expression in zebrafish, which is not maternal, and could indicate the involvement of carp *shh* in pattern formation before the zygotic genome activation (Wang et al. 2007).

Maternal Factors Involved in Gastrulation

Maternal interferon regulatory factor 6 (Irf6) plays a role in governing the segregation of blastomeres into an early epithelial fate and was shown to be required for the differentiation of the enveloping layer (EVL) in zebrafish embryos (Sabel et al. 2009). The maternal origin of zebrafish *irf6* was evidenced by its observation by in situ hybridization from 2 hpf, and the injection of a RNA encoding a dominant inhibitory Irf6 protein caused severe gastrulation defects. Indeed, the injected embryos showed that Irf6 is required for epiboly in the three germ layers, and

markers of EVL were lost. Finally, the Irf6-depleted embryos died at gastrula stage.

Pou5f1/Oct4 is a well-known transcription factor required for pluripotency of embryonic stem cells in mammals (reviewed in Boiani and Scholer 2005; Ralston and Rossant 2005). Recently, Lachnit and coworkers used zebrafish *pou5f1*-deficient maternal and zygotic *spiel ohne grenzen* (MZ*spg*) mutant embryos, to reassess in detail the gastrulation defects manifested by those embryos, and found that the three embryonic lineages presented alterations of the cytoskeleton, which contributed to a severe delay in epiboly progression (Lachnit et al. 2008).

Maternal Factors Involved in Neurogenesis

Brg1, a member of the SWI/SNF chromatin remodeling complex, is a maternal factor in zebrafish, as evidenced by its presence at the 1-cell stage (Gregg et al. 2003). This was confirmed by whole mount in situ hybridization staining from the 1-cell stage onward (Eroglu et al. 2006). The embryonic requirements of Brg1 were studied using a double approach (specific MO mediated knockdown, and heterozygote intercrosses of Brg1-deficient zebrafish (*yng*)), showing that Brg1 is critical in brain development and neural crest induction and differentiation (Eroglu et al. 2006).

Maternal Factors Involved in Somitogenesis

Misty somites (*mys*), a maternal-effect factor, has been shown to be involved in a fairly late event in embryonic development in the zebrafish, namely the somite boundary formation (Kotani and Kawakami 2008). The reduced activity of *mys* led to a pleiotropic effect: an obscure somite boundary at the 6-somite stage (12–13 hpf), the disappearance of the somite boundary at the 21-somite stage (20 hpf), and a curved phenotype at 26 hpf. Nevertheless, microinjection experiments performed using MOs targeted against translation initiation (ATG-MOs) or against splice donor MOs (SD-MOs) showed that the earlier phenotypes (at 6 and 12–13 hpf) were caused by a decrease in the maternal Mys activity, while zygotic Mys activity is thought to be responsible for the curved phenotype.

Transcriptomic Analysis of Oocyte Quality in Fish

Maternal mRNAs have been studied in unfertilized eggs of varying quality. In teleost species, postovulatory ageing can induce major egg quality problems (Bonnet et al. 2007a; Bobe and Labbe 2010; Fig. 11.2). In rainbow trout, postovulatory ageing was used as an experimental factor to induce a decrease in egg quality. Using real-time PCR, the maternal mRNA levels of 40 different genes, selected on the basis of the biological functions of corresponding proteins, were analyzed during postovulatory ageing and in eggs of varying quality (Aegerter et al. 2005). Low-quality eggs were characterized by significantly lower embryonic survival at eyed stage, hatching, and yolk-sac resorption, and by an increased incidence of embryonic malformations. This study showed that postovulatory ageing was associated with changes of the abundance of specific maternal mRNAS. During postovulatory ageing, nucleoplasmin (*npm2*), ferritin H, tubulin beta, *jnk1*, cyclin A1, cyclin A2, cathepsin Z, and *igf2* exhibited significant changes in mRNA abundance. The mRNA abundance of *npm2* exhibited a sharp decrease throughout the postovulatory period. In addition, seven transcripts exhibited a differential abundance between eggs of good and poor quality (Table 11.2). Low-quality eggs were characterized by low levels of *npm2*, tubulin beta, and *igf1* transcripts while keratins 8 and 18 and prostaglandin synthase 2 transcripts were more abundant in high-quality eggs. Using the same experimental model (i.e., postovulatory ageing as a tool to induce egg quality defects), an earlier study had reported that mRNA levels of IGF receptor were negatively correlated with developmental abnormalities in the larval stages (Aegerter et al. 2004). More recently, a microarray approach was used to analyze the transcriptome of rainbow trout eggs of varying quality (Bonnet et al. 2007b) and using a thorough analysis of egg quality based on both embryonic success and malformation abnormalities (Bonnet et al. 2007a). This study resulted in the identification of 31 genes showing a differential mRNA abundance in unfertilized rainbow trout eggs of varying quality. Among those genes, prohibitin 2 (phb2) was of particular interest. As shown by real-time PCR, phb2 mRNA abundance was negatively correlated with developmental success assessed by

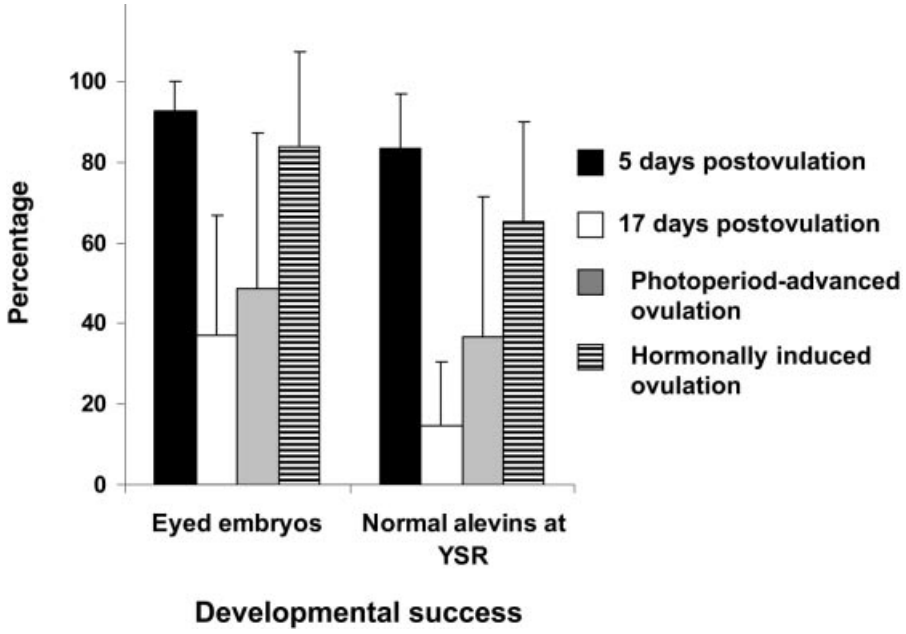


Figure 11.2. Developmental success assessed through embryonic survival at eyed stage and percentage of normal alevins at yolk-sac resorption (YSR) after natural ovulation, natural ovulation followed by a 17-day postovulatory ageing, photoperiod-advanced spawning, and hormonally induced spawning. Both parameters are expressed as a percentage of the number of eggs subjected to in vitro fertilization. (Redrawn from Bonnet et al. 2007a.)

the percentage of normal (i.e., without any noticeable malformation) alevins reaching yolk-scar resorption stage (Table 11.2). Prohibitins are highly conserved proteins found in multiple cellular compartments of eukaryotic cells. Prohibitins appear to be involved in a wide variety of cellular functions, including cell proliferation and modulation of transcriptional activity (Mishra et al. 2006). In the same transcriptomic study, carried out in rainbow trout, it was shown that the maternal mRNA of ribosomal protein L24 was overabundant when ovulation was hormonally triggered using a GnRH analog (Bonnet et al. 2007b). In zebrafish, it was previously reported that *rpl64* gene was essential for early embryonic development and that a mutation of this gene resulted in small head/eye phenotype (Golling et al. 2002). Interestingly, Bonnet and coworkers noticed that many rainbow trout embryos originating from eggs of hormonally induced females exhibited small eyes at eyeing stage (Bonnet et al. 2007b).

CONCLUSION

In summary, the molecular picture of the fish oocyte remains fuzzy even though a great deal of information is available from many separate candidate gene studies. In addition, several genomic-based analyses have generated large amounts of data that have been deposited in public databases (e.g., Gene Expression Omnibus, the microarray data repository of the National Center for Biotechnology Information). As well, a tool recently developed by Chan and colleagues (Chan et al. 2009) integrates dispersed data on expression, localization, and interactions from several genes and signaling pathways. The data gathered was used to develop network models of the gene regulatory interactions underlying early zebrafish development and can be accessed at www.zebrafishgrns.org. The identities of several abundant maternal mRNAs (e.g., lectin family members) have been revealed by several studies. However, a meta-analysis of maternal transcriptome

Table 11.2. Maternal mRNAs for which a Differential Abundance was Evidenced Using QPCR in Eggs of Varying Quality.

Symbol	Encoded Protein	Expression Pattern	Experimental Factor Used to Induced Egg Quality Defects	Parameter Used to Assess Egg Quality	Reference
ifg2	Insulin-like growth factor 2	Lower mRNA levels in low quality eggs	Postovulatory ageing	Survival at eyed stage	Aegerter et al. 2004
igf1	Insulin-like growth factor 1				Aegerter et al. 2004, 2005
npm2	Nucleoplasmin				Aegerter et al. 2005
tubb	Tubulin beta				Aegerter et al. 2005
krt8	Keratin 8	Higher mRNA levels in low quality eggs	Postovulatory ageing	Survival at eyed stage	Aegerter et al. 2005
krt18	Keratin 18				Aegerter et al. 2005
ctsz	Cathepsin Z				Aegerter et al. 2005
ptgs2	Prostaglandin synthase 2				Aegerter et al. 2005
phb2	Prohibitin 2		Spawning induction or photoperiod	Percentage of normal alevins at yolk-sac resorption	Bonnet et al. 2007b

would be required to obtain a clearer molecular portrait of the fish oocyte. This molecular picture of the oocyte is partially completed with the recent finding of RNAs, which are themselves involved in mRNA regulation. They are siRNA, microRNA, and a separate class of germ-cell-specific small RNA molecules named Piwi-interacting RNAs all interacting with proteins from the Argonaute family (like is Piwi), which may be maternal (Houwing et al. 2007). Finally, it seems clear that maternal mRNAs contribute not only to early embryonic steps occurring prior to MBT but also to critical embryonic events occurring after MBT. The maternal mRNAs are therefore likely to play a major role in the control of the egg ability to develop, once fertilized, into a normal embryo. This is further supported by recent transcriptomic analyses of the unfertilized fish eggs, indicating that maternal mRNA accumulation within the egg is subjected to environmental influence and sometimes associated with differential developmental capacities (Bonnet et al. 2007b). The molecular portrait of the developmentally competent fish oocyte is, however, far from being complete and further genomic and functional studies are still required to fully understand the contribution of maternal mRNAs to the developmental competence of the oocyte.

REFERENCES

Aegerter S, Jalabert B, and Bobe J. 2004. Messenger RNA stockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulin-like growth factor receptor Ib, and p53 in the rainbow

trout oocyte in relation with developmental competence. Mol Reprod Dev. 67: 127–135.

Aegerter S, Jalabert B, and Bobe J. 2005. Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. Mol Reprod Dev. 72: 377–385.

Aizawa K, Shimada A, Naruse K, Mitani H, and Shima A. 2003. The medaka midblastula transition as revealed by the expression of the paternal genome. Gene Expr Patterns. 3: 43–47.

Aoki TO, Mathieu J, Saint-Etienne L, Rebagliati MR, Peyrieras N, and Rosa FM. 2002. Regulation of nodal signalling and mesendoderm formation by TARAM-A, a TGFbeta-related type I receptor. Dev Biol. 241: 273–288.

Bai J, Solberg C, Fernandes JM, and Johnston IA. 2007. Profiling of maternal and developmental-stage specific mRNA transcripts in Atlantic halibut *Hippoglossus hippoglossus*. Gene. 386: 202–210.

Barker N. 2008. The canonical Wnt/beta-catenin signalling pathway. Meth Mol Biol. 468: 5–15.

Bellipanni G, Varga M, Maegawa S, Imai Y, Kelly C, Myers AP, Chu F, Talbot WS, and Weinberg ES. 2006. Essential and opposing roles of zebrafish beta-catenins in the formation of dorsal axial structures and neurectoderm. Development. 133: 1299–1309.

Black SD and Gerhart JC. 1986. High-frequency twinning of *Xenopus laevis* embryos from eggs centrifuged before first cleavage. Dev Biol. 116: 228–240.

Blaser H, Eisenbeiss S, Neumann M, Reichman-Fried M, Thisse B, Thisse C, and Raz E. 2005. Transition from non-motile behaviour to directed migration during early PGC development in zebrafish. J Cell Sci. 118: 4027–4038.

Bobe J and Labbe C. 2010. Egg and sperm quality in fish. Gen Comp Endocrinol. 165(3): 535–548.

Boiani M and Scholer HR. 2005. Regulatory networks in embryo-derived pluripotent stem cells. Nat Rev Mol Cell Biol. 6: 872–884.

Bonnet E, Fostier A, and Bobe J. 2007a. Characterization of rainbow trout egg quality: a case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. Theriogenology. 67: 786–794.

Bonnet E, Fostier A, and Bobe J. 2007b. Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics. 8: 55.

Braat AK, van de WS, Korving J, and Zivkovic D. 2001. A zebrafish vasa morphant abolishes vasa protein but does not affect the establishment of the germline. Genesis. 30: 183–185.

Brooks S, Tyler CR, and Sumpter JP. 1997. Egg quality in fish: what makes a good egg? Rev Fish Biol Fisher. 7: 387–416.

Chan TM, Longabaugh W, Bolouri H, Chen HL, Tseng WF, Chao CH, Jang TH, Lin YI, Hung SC, Wang HD, and Yuh CH. 2009. Developmental gene regulatory networks in the zebrafish embryo. Biochim Biophys Acta. 1789: 279–298.

Chen S and Kimelman D. 2000. The role of the yolk syncytial layer in germ layer patterning in zebrafish. Development. 127: 4681–4689.

Dosch R, Wagner DS, Mintzer KA, Runke G, Wiemelt AP, and Mullins MC. 2004. Maternal control of vertebrate development before the midblastula transition: mutants from the zebrafish I. Dev Cell. 6: 771–780.

Eddy EM. 1975. Germ plasm and the differentiation of the germ cell line. Int Rev Cytol. 43: 229–280.

Eisen JS and Smith JC. 2008. Controlling morpholino experiments: don't stop making antisense. Development. 135: 1735–1743.

Eroglu B, Wang G, Tu N, Sun X, and Mivechi NF. 2006. Critical role of Brg1 member of the SWI/SNF chromatin remodeling complex during neurogenesis and neural crest induction in zebrafish. Dev Dyn. 235: 2722–2735.

Ferg M, Sanges R, Gehrig J, Kiss J, Bauer M, Lovas A, Szabo M, Yang L, Straehle U, Pankratz MJ, Olasz F, Stupka E, and Muller F. 2007. The TATA-binding protein regulates maternal mRNA degradation and differential zygotic transcription in zebrafish. EMBO J. 26: 3945–3956.

Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, and Schier AF. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science. 312: 75–79.

Golling G, Amsterdam A, Sun Z, Antonelli M, Maldonado E, Chen W, Burgess S, Haldi M, Artzt K, Farrington S, Lin SY, Nissen RM, and Hopkins N. 2002. Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. Nat Genet. 31: 135–140.

Gore AV, Maegawa S, Cheong A, Gilligan PC, Weinberg ES, and Sampath K. 2005. The zebrafish dorsal axis is apparent at the four-cell stage. Nature. 438: 1030–1035.

Gregg RG, Willer GB, Fadool JM, Dowling JE, and Link BA. 2003. Positional cloning of the young mutation identifies an essential role for the Brahma

chromatin remodeling complex in mediating retinal cell differentiation. Proc Natl Acad Sci USA. 100: 6535–6540.

Hagos EG, Fan X, and Dougan ST. 2007. The role of maternal Activin-like signals in zebrafish embryos. Dev Biol. 309: 245–258.

Hashimoto Y, Maegawa S, Nagai T, Yamaha E, Suzuki H, Yasuda K, and Inoue K. 2004. Localized maternal factors are required for zebrafish germ cell formation. Dev Biol. 268: 152–161.

Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den EH, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RH, Hannon GJ, Draper BW, and Ketting RF. 2007. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. Cell. 129: 69–82.

Iwamatsu T. 2004. Stages of normal development in the medaka *Oryzias latipes*. Mech Dev. 121: 605–618.

Jia S, Ren Z, Li X, Zheng Y, and Meng A. 2008. smad2 and smad3 are required for mesendoderm induction by transforming growth factor-/nodal signals in zebrafish. J Biol Chem. 283: 2418–2426.

Kane DA and Kimmel CB. 1993. The zebrafish midblastula transition. Development. 119: 447–456.

Kane DA, McFarland KN, and Warga RM. 2005. Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly. Development. 132: 1105–1116.

Kimelman D. 2006. Squinting at the zebrafish axis. Dev Cell. 10: 6–7.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, and Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn. 203: 253–310.

Knaut H, Pelegri F, Bohmann K, Schwarz H, and Nusslein-Volhard C. 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. J Cell Biol. 149: 875–888.

Knaut H, Steinbeisser H, Schwarz H, and Nusslein-Volhard C. 2002. An evolutionary conserved region in the vasa 3'UTR targets RNA translation to the germ cells in the zebrafish. Curr Biol. 12: 454–466.

Knoll-Gellida A, Andre M, Gattegno T, Forgue J, Admon A, and Babin PJ. 2006. Molecular phenotype of zebrafish ovarian follicle by serial analysis of gene expression and proteomic profiling, and comparison with the transcriptomes of other animals. BMC Genomics. 7: 46.

Kofron M, Puck H, Standley H, Wylie C, Old R, Whitman M, and Heasman J. 2004. New roles for FoxH1 in patterning the early embryo. Development. 131: 5065–5078.

Koprunner M, Thisse C, Thisse B, and Raz E. 2001. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev. 15: 2877–2885.

Kotani T and Kawakami K. 2008. Misty somites, a maternal effect gene identified by transposon-mediated insertional mutagenesis in zebrafish that is essential for the somite boundary maintenance. Dev Biol. 316: 383–396.

Kumar A, Novoselov V, Celeste AJ, Wolfman NM, ten Dijke P, and Kuehn MR. 2001. Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J Biol Chem. 276: 656–661.

Kwon JY, Prat F, Randall C, and Tyler CR. 2001. Molecular characterization of putative yolk processing enzymes and their expression during oogenesis and embryogenesis in rainbow trout (*Oncorhynchus mykiss*). Biol Reprod. 65: 1701–1709.

Lachnit M, Kur E, and Driever W. 2008. Alterations of the cytoskeleton in all three embryonic lineages contribute to the epiboly defect of Pou5f1/Oct4 deficient MZspg zebrafish embryos. Dev Biol. 315: 1–17.

Lecuyer E, Yoshida H, Parthasarathy N, Alm C, Babak T, Cerovina T, Hughes TR, Tomancak P, and Krause HM. 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell. 131: 174–187.

Li W, Deng F, Wang H, Zhen Y, Xiang F, Sui Y, and Li J. 2006. Germ cell-less expression in zebrafish embryos. Dev Growth Differ. 48: 333–338.

Liu F, Kohlmeier S, and Wang CY. 2008. Wnt signaling and skeletal development. Cell Signal. 20: 999–1009.

Lo J, Lee S, Xu M, Liu F, Ruan H, Eun A, He Y, Ma W, Wang W, Wen Z, and Peng J. 2003. 15000 unique zebrafish EST clusters and their future use in microarray for profiling gene expression patterns during embryogenesis. Genome Res. 13: 455–466.

Mathavan S, Lee SG, Mak A, Miller LD, Murthy KR, Govindarajan KR, Tong Y, Wu YL, Lam SH, Yang H, Ruan Y, Korzh V, Gong Z, Liu ET, and Lufkin T. 2005. Transcriptome analysis of zebrafish embryogenesis using microarrays. PLoS Genet. 1: 260–276.

Mendelsohn BA and Gitlin JD. 2008. Coordination of development and metabolism in the pre-midblastula transition zebrafish embryo. Dev Dyn. 237: 1789–1798.

Mishra S, Murphy LC, and Murphy LJ. 2006. The Prohibitins: emerging roles in diverse functions. J Cell Mol Med. 10: 353–363.

Mizuno T, Yamaha E, Kuroiwa A, and Takeda H. 1999. Removal of vegetal yolk causes dorsal deficencies and impairs dorsal-inducing ability of the yolk cell in zebrafish. Mech Dev. 81: 51–63.

Muller K, Thisse C, Thisse B, and Raz E. 2002. Expression of a linker histone-like gene in the primordial germ cells in zebrafish. Mech Dev. 117: 253–257.

Nganvongpanit K, Muller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, and Tesfaye D. 2006. Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence-specific double-stranded RNA. Reproduction. 131: 861–874.

Ober EA and Schulte-Merker S. 1999. Signals from the yolk cell induce mesoderm, neuroectoderm, the trunk organizer, and the notochord in zebrafish. Dev Biol. 215: 167–181.

Pei W, Noushmehr H, Costa J, Ouspenskaia MV, Elkahloun AG, and Feldman B. 2007a. An early requirement for maternal FoxH1 during zebrafish gastrulation. Dev Biol. 310: 10–22.

Pei W, Williams PH, Clark MD, Stemple DL, and Feldman B. 2007b. Environmental and genetic modifiers of squint penetrance during zebrafish embryogenesis. Dev Biol. 308: 368–378.

Pelegri F. 2003. Maternal factors in zebrafish development. Dev Dyn. 228: 535–554.

Pogoda HM, Solnica-Krezel L, Driever W, and Meyer D. 2000. The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of nodal signaling required for organizer formation. Curr Biol. 10: 1041–1049.

Ralston A and Rossant J. 2005. Genetic regulation of stem cell origins in the mouse embryo. Clin Genet. 68: 106–112.

Ramasamy S, Wang H, Quach HN, and Sampath K. 2006. Zebrafish Staufen1 and Staufen2 are required for the survival and migration of primordial germ cells. Dev Biol. 292: 393–406.

Raz E. 2000. The function and regulation of vasa-like genes in germ-cell development. Genome Biol. 1(3): reviews1017.1–1017.6.

Reversade B, Kuroda H, Lee H, Mays A, and De Robertis EM. 2005. Depletion of Bmp2, Bmp4, Bmp7 and Spemann organizer signals induces massive brain formation in *Xenopus* embryos. Development. 132: 3381–3392.

Sabel JL, d'Alencon C, O'Brien EK, Otterloo EV, Lutz K, Cuykendall TN, Schutte BC, Houston DW, and Cornell RA. 2009. Maternal interferon regulatory factor 6 is required for the differentiation of primary superficial epithelia in *Danio* and *Xenopus* embryos. Dev Biol. 325: 249–262.

Scharf SR and Gerhart JC. 1983. Axis determination in eggs of *Xenopus laevis*: a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation. Dev Biol. 99: 75–87.

Schier AF and Talbot WS. 2005. Molecular genetics of axis formation in zebrafish. Annu Rev Genet. 39: 561–613.

Scholz S, Domaschke H, Kanamori A, Ostermann K, Rodel G, and Gutzeit HO. 2004. Germ cell-less expression in medaka (*Oryzias latipes*). Mol Reprod Dev. 67: 15–18.

Schyth BD. 2008. RNAi-mediated gene silencing in fishes? J Fish Biol. 72: 1890–1906.

Shi Y and Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 113: 685–700.

Shinomiya A, Tanaka M, Kobayashi T, Nagahama Y, and Hamaguchi S. 2000. The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, *Oryzias latipes*. Dev Growth Differ. 42: 317–326.

Strasser MJ, Mackenzie NC, Dumstrei K, Nakkrasae LI, Stebler J, and Raz E. 2008. Control over the morphology and segregation of zebrafish germ cell granules during embryonic development. BMC Dev Biol. 8: 58.

Sun Z, Jin P, Tian T, Gu Y, Chen YG, and Meng A. 2006. Activation and roles of ALK4/ALK7-mediated maternal TGFbeta signals in zebrafish embryo. Biochem Biophys Res Commun. 345: 694–703.

Suzuki H, Maegawa S, Nishibu T, Sugiyama T, Yasuda K, and Inoue K. 2000. Vegetal localization of the maternal mRNA encoding an EDEN-BP/Bruno-like protein in zebrafish. Mech Dev. 93: 205–209.

Takashima S, Shimada A, Kobayashi D, Yokoi H, Narita T, Jindo T, Kage T, Kitagawa T, Kimura T, Sekimizu K, Miyake A, Setiamarga DH, Murakami R, Tsuda S, Ooki S, Kakihara K, Hojo M, Naruse K, Mitani H, Shima A, Ishikawa Y, Araki K, Saga Y, and Takeda H. 2007. Phenotypic analysis of a novel chordin mutant in medaka. Dev Dyn. 236: 2298–2310.

Tata JR. 1986. Coordinated assembly of the developing egg. Bioessays. 4: 197–201.

Theusch EV, Brown KJ, and Pelegri F. 2006. Separate pathways of RNA recruitment lead to the

compartmentalization of the zebrafish germ plasm. Dev Biol. 292: 129–141.

Van Raay TJ, Coffey RJ, and Solnica-Krezel L. 2007. Zebrafish Naked1 and Naked2 antagonize both canonical and non-canonical Wnt signaling. Dev Biol. 309: 151–168.

Wagner DS, Dosch R, Mintzer KA, Wiemelt AP, and Mullins MC. 2004. Maternal control of development at the midblastula transition and beyond: mutants from the zebrafish II. Dev Cell. 6: 781–790.

Wang N, Sun YH, Liu J, Wang YP, and Zhu ZY. 2007. Molecular characterization of common carp (*Cyprinus carpio*) sonic hedgehog and discovery of its maternal expression. Dev Genes Evol. 217: 299–305.

Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, and Raz E. 2003. Dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol. 13: 1429–1434.

Weidinger G, Wolke U, Köprunner M, Klinger M, and Raz E. 1999. Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. Development. 126(23): 5295–5307.

Wen C, Zhang Z, Ma W, Xu M, Wen Z, and Peng J. 2005. Genome-wide identification of female-enriched genes in zebrafish. Dev Dyn. 232: 171–179.

Whitman M. 2001. Nodal signaling in early vertebrate embryos: themes and variations. Dev Cell. 1: 605–617.

Wibrand K and Olsen LC. 2002. Linker histone H1M transcripts mark the developing germ line in zebrafish. Mech Dev. 117: 249–252.

Widelitz R. 2005. Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors. 23: 111–116.

Wolke U, Weidinger G, Koprunner M, and Raz E. 2002. Multiple levels of posttranscriptional control lead to germ line-specific gene expression in the zebrafish. Curr Biol. 12: 289–294.

Xu H, Li M, Gui J, and Hong Y. 2007. Cloning and expression of medaka dazl during embryogenesis and gametogenesis. Gene Expr Patterns. 7: 332–338.

Zhao XF, Fjose A, Larsen N, Helvik JV, and Drivenes O. 2008. Treatment with small interfering RNA affects the microRNA pathway and causes unspecific defects in zebrafish embryos. FEBS J. 275: 2177–2184.

Chapter 12

Application of Fish Stem Cell Technology to Aquaculture and Marine Biotechnology

Ten-Tsao Wong and Paul Collodi

INTRODUCTION

Stem cells are characterized by the ability to self-renew by mitotic cell division and to differentiate into a number of different cell types. Stem cell cultures provide a valuable model for both in vitro and in vivo studies of growth and differentiation (Liu et al. 1993; Zhang et al. 1995). Mouse pluripotent embryonic stem (ES) cell cultures are routinely used to introduce genetic alterations into the germ line, providing an efficient approach to the study of gene function during development and growth. To pursue this approach, genetically modified mouse ES cells are selected and grown in culture and the genetic alteration is then transferred to the animal when the cultured cells are transplanted into a recipient embryo where they contribute to the germ cell lineage (Doetschman et al. 1987; Capecchi 1989). In addition to ES cells, another pluripotent stem cell type that has been used for gene transfer studies is the embryonic germ (EG) cell. When primordial germ cells (PGCs) are isolated from the mammalian or avian developing gonad and maintained in culture, the cells begin to exhibit pluripotent characteristics and are referred to as EG cells (Matsui et al. 1992; Park and Han 2000; Park et al. 2003). The conversion of PGCs to the pluripotent EG cell phenotype in culture has provided a model for in vitro investigation of cellular reprogramming (Durcova-Hills et al. 2006). In addition, the EG cell cultures have been used to generate transgenic mice (Piedrahita et al. 1998) and chickens (van de Lavoir et al. 2006) by germ-line chimera production.

On the basis of the wide range of studies that have been pursued using stem cell cultures from mammalian and avian systems, it is reasonable to expect that stem cell technology also holds tremendous potential for a variety of applications in the fields of aquaculture and marine biotechnology. To develop this potential, several laboratories have been working to establish stem cell cultures from aquatic species (Bejar et al. 2002; Chen et al. 2003a, b; Shikina et al. 2008). Much of the research directed toward developing this technology has been done using model fish species such as medaka (Wakamatsu et al. 1994; Hong et al. 1996) and zebrafish (Sun et al. 1995; Ma et al. 2001; Kurita and Sakai 2004). Once the technology is established, it may then be applied to commercially important aquaculture

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

species. Our laboratory and others (Ma et al. 2001; Fan et al. 2004a, c, 2008; Kurita et al. 2004; Kurita and Sakai 2004) have been working to derive zebrafish stem cell cultures that will be suitable for gene-transfer studies. In our laboratory, research has been directed toward establishing methods to derive pluripotent ES cell and PGC cultures from zebrafish embryos. To date, we have found that the ES cell lines derived from zebrafish embryos remain germ line competent for multiple passages in culture (Ma et al. 2001; Fan et al. 2004a, c); however, the low efficiency of germ line transmission using the ES cell cultures led us to develop methods for the derivation of zebrafish PGC cultures (Fan et al. 2008). Working toward a similar goal, Kurita and Sakai have developed stem cell cultures from zebrafish spermatogonia that proliferate and generate mature sperm in culture (Kurita and Sakai 2004). The spermatogonial cell cultures were used to generate a transgenic line of zebrafish by retroviral infection of the in vitro-produced sperm (Kurita et al. 2004).

In this chapter, we review the current status of research toward the development of stem cell technology in zebrafish and other fish species. In this context, we describe the derivation of zebrafish ES cell, PGC, and spermatogonial culture systems along with research toward the genetic manipulation of the cultured stem cells and the potential for applying this technology to other species that are important in marine biotechnology.

DERIVATION OF ZEBRAFISH ES CELL CULTURES

Since the zebrafish blastula-stage embryo (Fig. 12.1A) consists entirely of nondifferentiated cells, it is the optimal developmental stage for use in the initiation of ES cell cultures. Primary cultures were initiated from zebrafish blastulas on a growth-arrested feeder layer (Fig. 12.1B) consisting of rainbow trout spleen cells from the established RTS34st cell line (Ganassin and Bols 1999). When maintained on the feeder layer, the embryo cell cultures consist of multiple dense cell aggregates that possess a homogeneous appearance and lack any morphological indication of differentiation. As the embryo cells proliferate, the aggregates increase in size without losing their homogeneous, dense

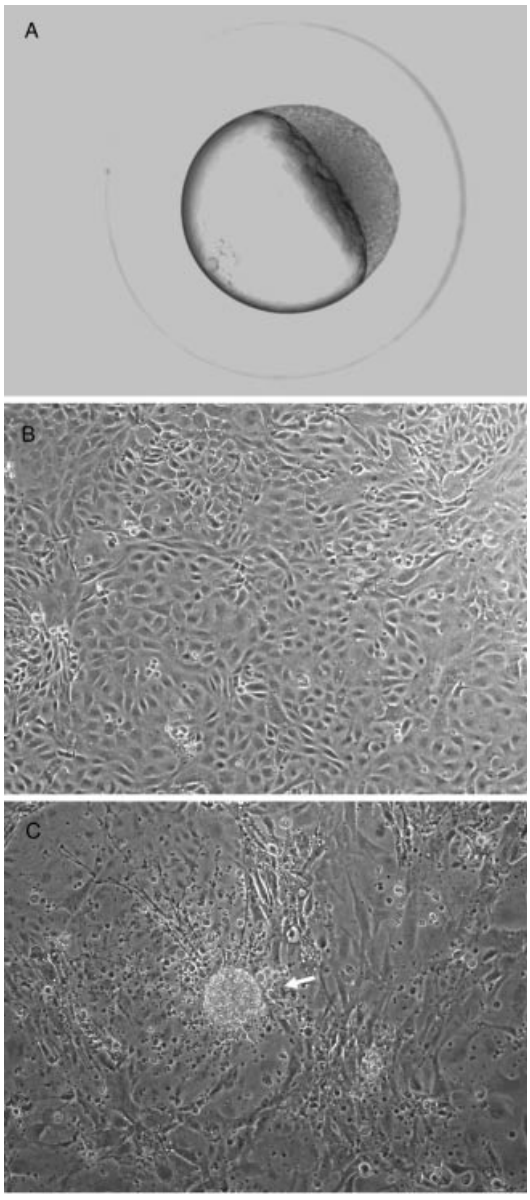


Figure 12.1. Blastula-stage zebrafish embryo (**A**). RTS34st cell line (Ganassin and Bols 1999) derived from the rainbow trout spleen and used as a feeder layer for zebrafish embryo cell cultures (**B**). Embryo cell culture derived from blastula showing ES-like morphology with aggregates of embryo cells possess a homogeneous appearance as indicated by arrow (**C**).

appearance (Fig. 12.1C). Using these methods, ES cell lines have been maintained for more than 200 population doublings in conditions that were shown to promote cell growth and preserve pluripotency of the cells (Ma et al. 2001). The culture conditions consist of LDF basal nutrient medium supplemented with human EGF, human bFGF, bovine insulin, FBS, trout serum, and growth-arrested RTS34st feeder cells (Ma et al. 2001). Factors that are produced by the RTS34st feeder cells maintain the pluripotency of the zebrafish ES cells, indicated by the presence of alkaline phosphatase activity and the capacity to form differentiated embryoid bodies in suspension culture.

In order to optimize the cell culture conditions with the goal of increasing the length of time that the ES cells can be maintained in vitro in a pluripotent condition, the cell culture medium was supplemented with mammalian growth and differentiation factors that are known to promote the pluripotency of mammalian and avian ES cells. Among the growth and differentiation factors that were evaluated on the zebrafish ES cell cultures were human and mouse leukemia inhibitory factor (LIF), mouse interleukin 6, human ciliary neurotrophic factor, mouse transforming growth factor-β1, and bone morphogenetic factor-4. None of these factors were found to enhance ES cell pluripotency based on the cells' level of alkaline phosphatase activity, their capacity to form differentiated embryoid bodies in suspension culture, or the cells' contribution to a host embryo following transplantation. Since stem cell factor (SCF) is required for the culture of pluripotent mammalian EG cells (Pesce et al. 1993; Geijsen et al. 2004), we obtained cDNAs encoding zebrafish SCFs, also known as kit ligands (Kitlga and Kitlgb; kind gift from Dr. Nathan Baharry, University of Pittsburgh), and expressed each of the recombinant proteins in RTS34st feeder cells. The results revealed that ES cells cultured in the presence of Kitlg maintained high levels of alkaline phosphatase activity and the capacity to form differentiated embryoid bodies for more passages than ES cells grown in the absence of the factor. Also, the addition of zebrafish Kitlg to the culture medium either as soluble purified recombinant protein synthesized in bacteria or expressed and secreted by the RTS34st feeder cells resulted in an increased growth rate of the ES cells.

The ultimate test of ES cell pluripotency is to evaluate the cells' ability to contribute to multiple tissues, including the germ line of a host embryo following transplantation. To examine the in vivo pluripotency of the zebrafish ES cell cultures, we initiated cultures from a transgenic line of zebrafish that expresses green fluorescence protein (GFP) throughout the body and possesses wild-type pigmentation (Higashijima et al. 1997). At each passage, the cultured ES cells were transplanted into host blastula-stage embryos obtained from the GASSI strain that lacks body pigmentation. Approximately, 50–100 cultured cells are injected into the cell mass of each recipient blastula. Three days after injection, the potential germ-line chimeras are identified by the presence of GFP-positive cells in the region of the gonad. To confirm germ-line chimerism, the candidate embryos were raised to sexual maturity and bred with non-injected GASSI mates and the resulting F1 generation was examined for expression of GFP throughout the body that was derived from the transplanted cells. Comparison of cultures at the first passage with those at passage 5 revealed that the frequency of germ line chimera production decreased from approximately 5% to less than 1% of the total number of injected embryos that survived to sexual maturity. Only two germ line chimeras were identified from approximately 250 fish that were transplanted with passage 5 ES cells (Fan et al. 2004b).

In addition to zebrafish, culture conditions for medaka ES cells have also been established (Wakamatsu et al. 1994; Hong et al. 1996). The medaka ES cells were grown for over 100 passages under defined culture conditions in the absence of feeder cells and exhibited characteristics of pluripotency, including alkaline phosphatase activity, embryoid body formation in suspension culture, and the ability to differentiate into a variety of cell types (Hong et al. 1996, 1998b). Other fish species from which ES-like cell lines have been established include gilthead seabream (Bejar et al. 1999), sea perch (Chen et al. 2003a), and red seabream (Chen et al. 2003b). The cell lines established from these species exhibit in vitro characteristics of pluripotency, including alkaline phosphatase activity and the capacity to differentiate into various cell types such as neuron-like cells and muscle cells in vitro (Bejar et al. 2002; Chen et al. 2003a). Several of the fish ES cell lines

have also been used to generate chimeras. Transplantation of ES cells from medaka and gilthead seabream into host embryos resulted in chimera production in 90% and 44% of the surviving embryos, respectively, with contribution of the transplanted ES cells to all three germ layers (Hong et al. 1998a; Bejar et al. 2002). In addition to ES cell lines, stem cell cultures have also been established from zebrafish (Kurita and Sakai 2004) and trout (Shikina et al. 2008) spermatogonia. In zebrafish, the spermatogonial stem cells were initiated and maintained on a feeder layer of tumor-like testis-derived ZtA6 cells. By using different ZtA6 lines, the spermatogonia could be either maintained as proliferating stem cells or induced to differentiate into mature sperm that could then be used to generate transgenic fish in vitro fertilization (Kurita et al. 2004). In trout, spermatogonial stem cells were grown on feeder-free medium supplemented with FBS, insulin, trout embryonic extract, and FGF. The cultured trout spermatogonia were able to colonize recipient gonads following transplantation (Shikina et al. 2008). The establishment of stem cell lines from multiple fish species indicates that in the future, this technology may have a wide application to the aquaculture industry.

GENETIC MANIPULATION OF FISH ES CELL CULTURES

In order for fish stem cells to be useful for gene-targeting approaches, methods must be established to incorporate plasmid DNA into the cells in a targeted fashion by homologous recombination. We have examined the frequency of homologous recombination in zebrafish ES cell cultures and established methods to isolate colonies of targeted cells by positive–negative selection (Fan et al. 2006). For this work, targeting plasmids were designed to insert into the no tail gene (*ntl*) (Schulte-Merker et al. 1992; Schulte-Merker et al. 1994). The plasmids contained the *neo* expression cassette flanked by *ntl* 5' and 3' homologous regions. The 2.5 kb 5' homologous arm included the *ntl* promoter region and extended into the first exon and the 3.8 kb 3' homologous region spanned exons 1 through 9 (Fig. 12.2). All of the colonies that incorporated plasmid were selected in the presence of G418, and two different negative selection strategies were used to isolate the colonies that incorporated the plasmid by homologous recombination. The two selection methods involved the use of an expression cassette encoding either the red fluorescent protein (DsRed) or diphtheria toxin A-chain gene (*dt*) inserted outside of the *ntl* homologous region. When the DsRed cassette was used, after electroporation, cells that incorporated the plasmid by random insertion were DsRed positive, making it possible to avoid those colonies and instead manually select the DsRed-negative (targeted) colonies (Fan et al. 2006). Using the *dt* cassette for negative selection, the cells that contain random insertions express DT, which is lethal. Following G418 selection, the candidate targeted colonies (Neo+/DsRed− or Neo+/DT−) were selected (Fig. 12.2), expanded, and characterized by PCR and Southern blot analysis to confirm that targeted insertion of the plasmid had occurred (Fan et al. 2006).

In addition to targeting *ntl* in the cell cultures, our laboratory has also targeted other zebrafish genes that would generate dominant mutations when transferred into the embryo, making it possible to observe the phenotype in a heterozygous individual. Hag is an ortholog of mouse Dactylin and encodes an F-box/WD40-repeat protein that is involved in stripe pattern formation. Hag mutations generated by proviral insertion were dominant and resulted in obvious pigmentation pattern anomalies (Kawakami et al. 2000). In order to enhance visual selection, the *hag* targeting plasmid (pTG-pac-DsRed-DT-hag) was designed to contain a dt expression cassette as a negative selection marker and an expression cassette encoding a puromycin-DsRed fusion protein (Fig. 12.3A) instead of the *neo* cassette. This modification makes it possible to directly visualize DsRed+ cells that incorporate the plasmid by homologous recombination. Using this approach, our preliminary results showed that 1 out of 4 puromycin resistant/DT− and DsRed+ colonies incorporated the targeting plasmid by homologous recombination (Fig. 12.3B; Wong et al. unpublished results).

DERIVATION OF ZEBRAFISH PGC CULTURES

Fish PGCs are specified early during embryogenesis by the inheritance of asymmetrically localized

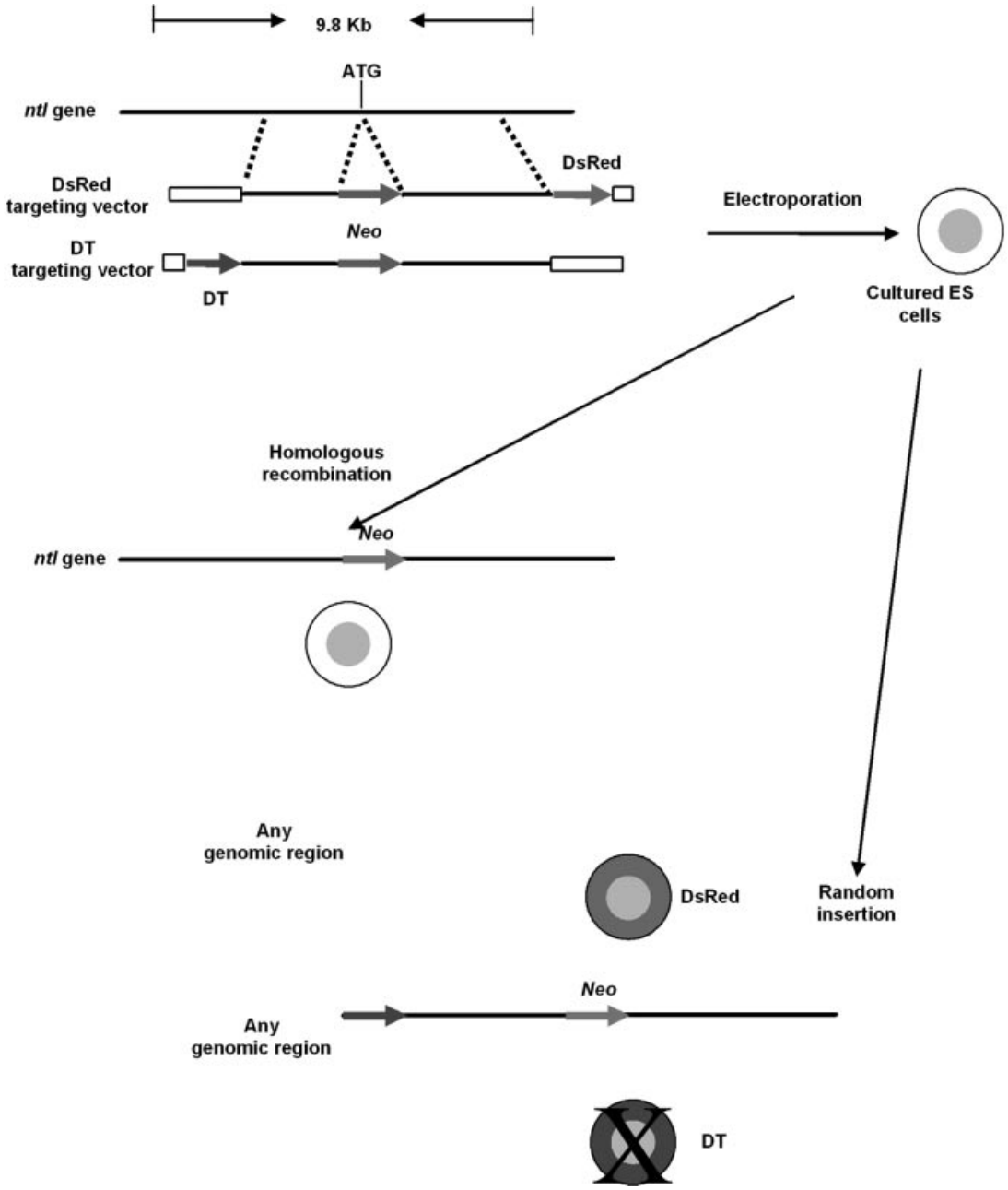


Figure 12.2. Targeted incorporation of pTG-Neo-DsRed-ntl or pTG-Neo-DT-ntl into zebrafish ES cells. The DsRed- or DT- colony is a candidate for homologous recombination. (For color detail, please see the color plate section.)

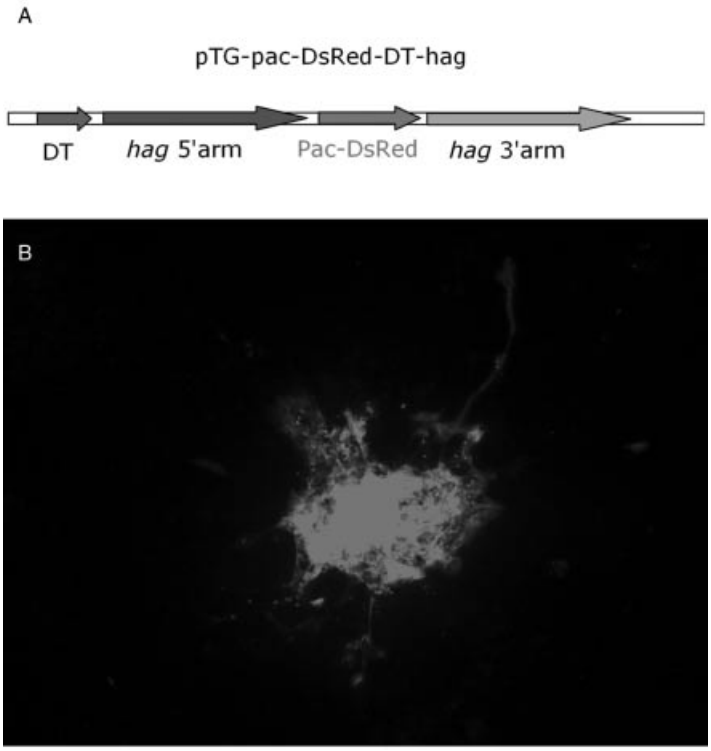


Figure 12.3. Diagram of pTG-pac-DsRed-DT-hag (**A**). In addition to puromycin (pac) selection, DsRed is used to visualize the colonies that had undergone targeted plasmid insertion by homologous recombination (**B**). (For color detail, please see the color plate section.)

maternal cytoplasm (Braat et al. 1999; Raz 2003; Herpin et al. 2007). During development, the PGCs proliferate and migrate to the developing gonad and give rise to gametes. Since the PGCs are committed to the germ cell lineage early in development, it is predicted that cell cultures comprising a homogeneous population of PGCs that were isolated from late-stage embryos would have a greater capacity than zebrafish ES cells to generate germ line chimeras following transplantation into a host embryo. Methods to establish homogeneous cultures of germ-line competent PGCs could be combined with the successful use of xenotransplantion of PGCs (see Chapter 14) to generate fertile chimeras (Takeuchi et al. 2003; Yoshizaki et al. 2005; Kobayashi et al. 2007; Okutsu et al. 2007; Saito et al. 2008). Such a system would make it possible to combine PGC-mediated gene manipulation with xenotransplantation to generate genetically modified fish. The PGCs that are genetically altered and selected in culture could be transplanted into a host of the same or closely related species to generate transgenic offspring.

In order to develop a PGC-mediated transgenic approach in fish, we established methods to derive cultures of proliferating zebrafish PGCs. The PGCs were visualized in culture by using a transgenic line of zebrafish that expresses DsRed under the control of the PGC-specific vasa promoter. Optimal growth of the PGCs was obtained when the cultures were initiated and maintained on growth-arrested RTS34st feeder cells that express recombinant zebrafish Kitlg. The culture medium was also supplemented with FGF and FBS. The requirement of Kitlg and FGF by the zebrafish PGCs is consistent with studies done with mouse (Matsui et al. 1992; Durcova-Hills et al. 2006) and chick PGC cultures (Park and Han 2000; Park et al. 2003). Our data showed that the addition of recombinant zebrafish Kitlga enhanced the average size of the PGC

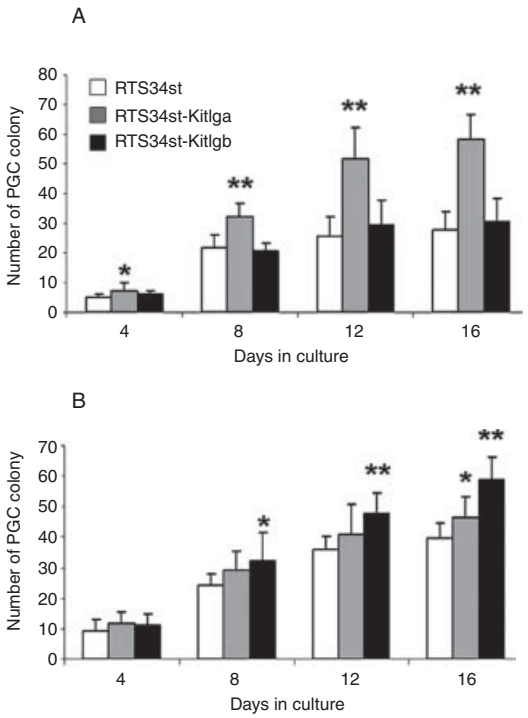


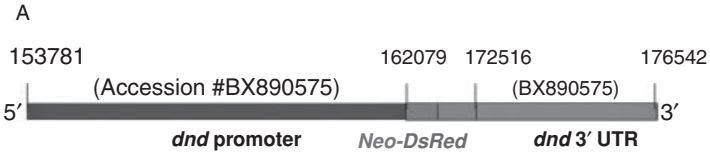
Figure 12.4. The effect of recombinant Kitlga and Kitlgb (**A**) and recombinant Sdf1a and Sdf1b (**B**) on the growth of zebrafish PGCs in culture. Cell cultures were initiated from 26-somite vasa::RFP embryos in wells containing RTS34st, RTS34st-Kitlga, or RTS34st-Kitlgb (**A**); and RTS34st, RTS34st-Sdf1a, or RTS34st-Sdf1b feeder cells. Number of RFP positive PGC colonies present in 4-, 8-, 12-, and 16-day-old cultures. All experiments were carried out in triplicate and the numbers represent the mean +/− SD of three separate experiments. Significant differences were analyzed by unpaired *t*-test with Minitab ($^{*}p < 0.05$; $^{**}p < 0.01$). Taken from Figure 4b and 5b of (Fan et al. 2008) and used with kind permission from Mary Ann Liebert, Inc.

colonies and also increased the overall number of colonies by 107.5% after 16 days in culture (Fig. 12.4A). The positive effect of Kitlga on zebrafish PGCs in culture was surprising since previous studies have shown that, unlike mammalian PGCs, in vivo the zebrafish germ cells do not express *kit,* and Kitlg is not required for normal PGC development (Parichy et al. 1999; Mellgren and Johnson 2005). Consistent with these studies, we have found that PGCs isolated from intact *vasa::RFP* embryos by fluorescence-activated cell sorting (FACS) do not express *kit*; however, once the cells are propagated in culture, they begin to express the *kit* (Fan et al. 2008). In vitro expression of the kit receptor makes it possible for the PGC cultures to respond to recombinant Kitlg added to the medium. Characterization of the cultures has also revealed that recombinant zebrafish stromal cell-derived factor 1 (Sdf1) enhances the survival or growth of zebrafish PGCs in culture (Fan et al. 2008). Zebrafish Sdf1 was added to the cultures through feeder cells that expressed the recombinant peptide. In mice and zebrafish, SDF1/Sdf1 serves as a chemokine that controls the directional migration of PGCs (Doitsidou et al. 2002; Molyneaux et al. 2003; Schier 2003; Raz 2004). Zebrafish have two *sdf1* genes that share approximately 70% identity (cDNA accession numbers:*sdf1a*, AY147915; *sdf1b*, AY347314). Consistent with our results showing an effect of Sdf1 on zebrafish PGCs in culture, mouse SDF1 has been shown to promote PGC survival in vivo (Molyneaux et al. 2003). Our results revealed that recombinant Sdf1b is more effective than Sdf1a in promoting zebrafish PGC growth in culture. In the presence of Sdf1b, the total number of PGCs in 16 day cultures increased 66.8% and the number of PGC colonies also increased by 48.8% (Fig. 12.4B). When cultures were supplemented with both Sdf1b and kitlga, the factors produced an additive effect by increasing the number of PGCs by 16.5% and the number of PGC colonies by 14.1% compared to cultures grown in the presence of zebrafish Kitlga alone (Fan et al. 2008).

For gene-targeting applications, it will be important to have available long-term homogeneous PGC cultures from fish species. Primary cell cultures initiated from whole fish embryos, such as the zebrafish cultures described above, are not suitable since only a small percentage of the cell population consists of PGCs. Initial attempts to address this problem by initiating cultures from isolated zebrafish *vasa::RFP* PGCs obtained by FACS were not successful. The isolated PGCs were not able to proliferate in culture most likely due to a requirement for the presence of

somatic cells in the primary culture to simulate the in vivo environment. To circumvent this problem, our laboratory has developed an approach involving the use of drug selection to obtain proliferating homogeneous zebrafish PGC colonies isolated from whole embryo primary cultures. The primary cultures were initiated from transgenic embryos that express plasmid (pdnd-Neo-DsRed) encoding a Neo-DsRed fusion protein under the control of the deadend (*dnd*) promoter and its 3'-UTR (Fig. 12.5A). Zebrafish dnd is specifically expressed in the PGCs through at least day 5 postfertilization and is required for germ cell survival in the embryo (Weidinger et al. 2003). Embryos at the 1-cell stage were injected with pdnd-Neo-DsRed and allowed to develop to the 12- to 15-somite stage before they were dissociated, and the cells were used to initiate cultures in the presence of growth-arrested RTS34st feeder cells that express zebrafish Kitlga. After approximately 6 weeks of G418 selection, drug-resistant and DsRed+ colonies were identified and expanded (Fig. 12.5B). The success of this approach may be due to the gradual elimination of somatic cells during G418 selection, thereby allowing the PGCs to proliferate and form colonies before all of the somatic cells were removed from the culture. Presently, the DsRed+ and G418-resistant colonies have been expanded and growing for more than 4 months. The results demonstrate that the cloned dnd promoter is able to drive stable expression of the fusion protein since the cells continue to express DsRed and proliferate in the presence of G418. After 4 months of drug selection, the cultures continue to express PGC markers, including *nanos*, *dnd*, and *vasa* (data not shown). To eliminate the need to initiate cultures from founder transgenic embryos, a stable *dnd::Neo-DsRed* transgenic line of zebrafish is currently being established in our laboratory (Wong et al., unpublished results). This transgenic line will make it possible to routinely obtain a large number of embryos for the initiation of PGC cultures.

An alternative strategy that has also been successful and may be applicable to a large variety of aquaculture species is to introduce into the embryos



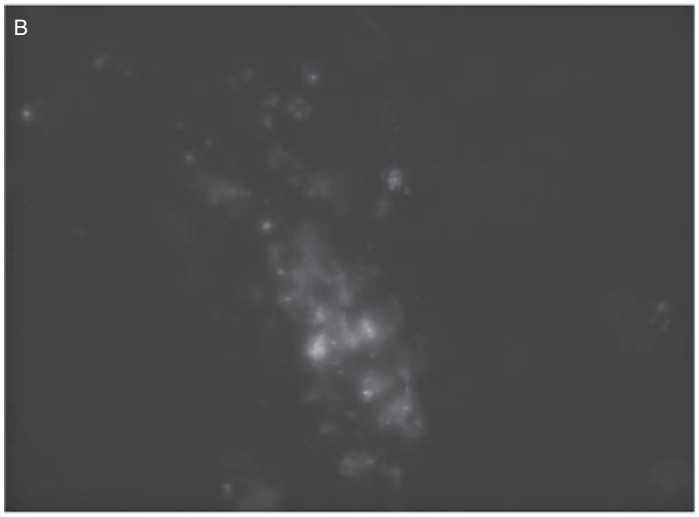


Figure 12.5. Diagram of pdnd-Neo-DsRed plasmid (**A**). The expression of Neo-DsRed fusion protein is controlled by the PGC specific promoter *dnd* and its 3′UTR. (**B**) The plasmid was injected into 1-cell embryos and later the embryos were used to initiate cultures. Following G418 selection, the DsRed expressing colonies were identified (**B**). (For color detail, please see the color plate section.)

mRNA encoding a puromycin-DsRed fusion protein along with the *nanos*-3'-UTR. The *nanos*-3'UTR directs expression of the fusion protein specifically in the germ cell lineage (Koprunner et al. 2001). Expression of the mRNA is stable for several days, a sufficient amount of time to initiate primary cultures and isolate colonies of PGCs in puromycin. By the time the injected mRNA is degraded and puromycin resistance is lost, all of the somatic cells have been eliminated from the culture. Since the PGC cultures that result from this approach are not harboring a transgene or expressing antibiotic resistance, the strategy may be more applicable to aquaculturally important species. To accomplish this approach in zebrafish, cDNA encoding with Pac-DsRed-nanos-3'UTR was constructed under the control of T7 promoter (Fig. 12.6A), and capped mRNA was synthesized. Approximately 40 pg of the mRNA was injected into 1- to 4-cell stage embryos. In initial experiments, the mRNA was injected into the *vasa::EGFP* transgenic line (Krovel and Olsen 2002), making it possible to examine the embryos for coexpression of both red and green fluorescence in the same cells, indicating that the mRNA was specifically incorporated and expressed in the PGCs (Fig. 12.6B). These embryos were collected, dissociated, and cell cultures were initiated on a feeder layer of growth-arrested RTS34st–kitlga. Puromycin (5 μg/mL final concentration) selection was maintained on the cultures as long as the cells continued to express DsRed fluorescence, indicating that the Pac-DsRed fusion protein was still present. PGC cultures derived from the injected embryos exhibited puromycin-resistance and stable DsRed expression up to 14 days in culture. The selected PGC colonies were expanded and continued to proliferate for multiple passages. After 3 months, RT-PCR analysis using RNA extracted from drug-selected PGC cultures revealed that the cells continued to express PGC-specific genes, including *vasa*, *dnd*, and *nanos*

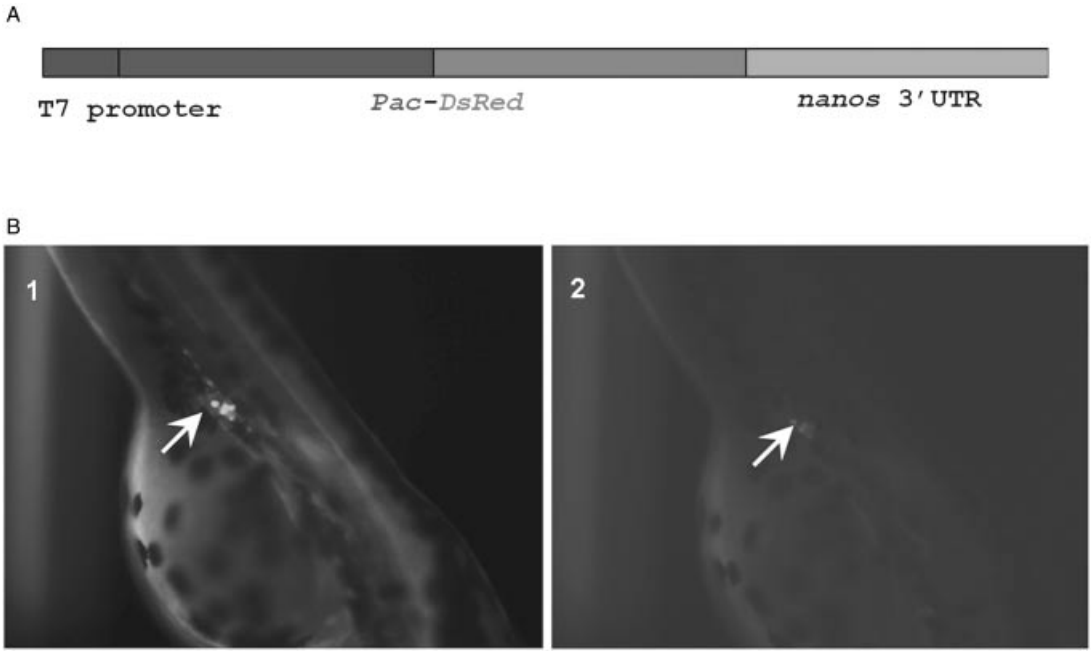


Figure 12.6. Diagram of T7-Pac-DsRed-nanos-3′UTR construct (**A**). A vasa::GFP embryo injected with *pac-DsRed-nanos*-3′UTR mRNA showing co-expression of green (1) and red (2) fluorescence specifically in PGCs (**B**). Taken from Figure 7 of Fan et al. (2008) and used with kind permission from Mary Ann Liebert, Inc. (For color detail, please see the color plate section.)

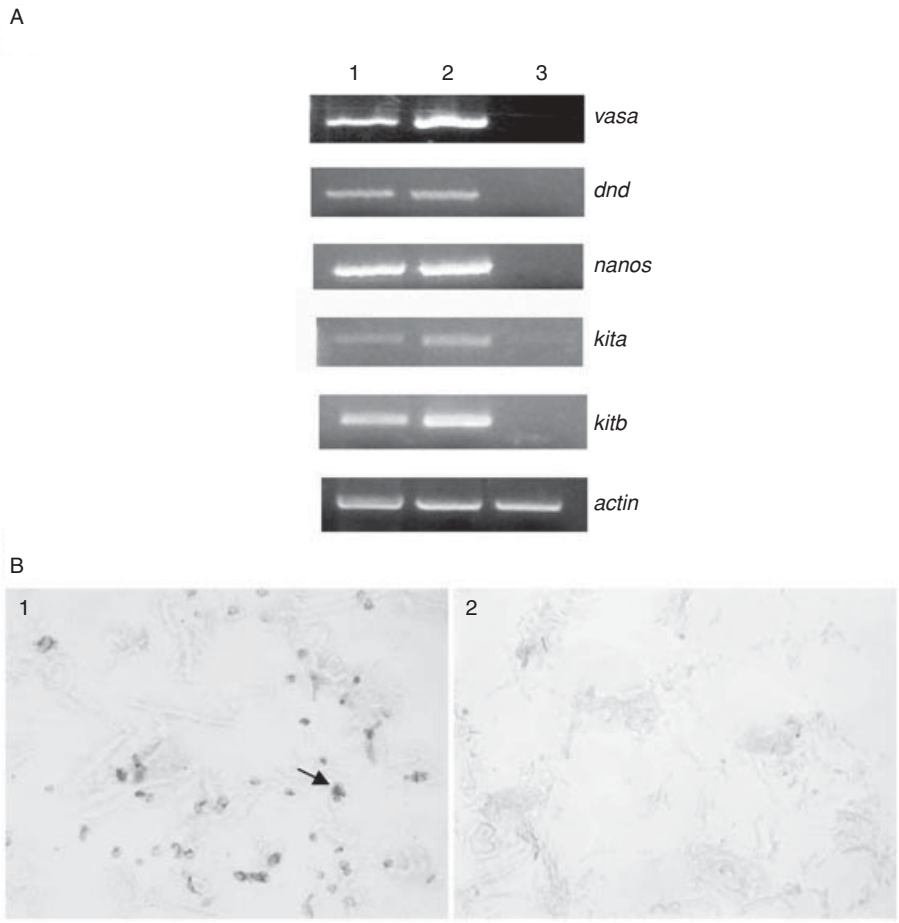


Figure 12.7. RT-PCR analysis of PGC-specific genes, vasa, dnd, and nanos using RNA extracted from drug-selected (*pac-DsRed-nanos*-3'UTR) PGC cultures (**A**). Lane 1, RT-PCR detection of PGC specific gene expression in passage 8 cells; lane 2, RNA isolated from 1-day-old embryo (positive control); lane 3, muscle RNA (negative control). In situ hybridization analysis of *vasa* expression in 3 month PGC cultures showing that a large percentage of the cells continue to express the gene. (1) Antisense and (2) sense probes (**B**). Taken from Figure 7 of Fan et al. (2008) and used with kind permission from Mary Ann Liebert, Inc.

(Fig. 12.7A). In situ hybridization with anti-*vasa* probe indicated that a large proportion of the late passage cells still expressed *vasa* (Fig. 12.7B).

GERM-LINE CHIMERA PRODUCTION FROM ZEBRAFISH PGC CULTURES

To evaluate the capacity of cultured zebrafish PGCs to generate germ line chimeras, multipassage cultures were transplanted into host embryos and germ line contribution was evaluated. The PGC cultures were derived by introducing the pdnd-Neo-DsRed construct into 1-cell stage embryos from the *actin::GFP* transgenic fish. Primary cultures were initiated from 15 somite-stage embryos in the presence of G418. The resulting PGC culture expressed GFP constitutively along with the Neo-DsRed

fusion protein under the control of the dnd promoter. PGCs from 4-month-old cultures were transplanted into GASSI recipient embryos and three days after transplantation, potential germ line chimeras were identified by the presence of DsRed-positive cells in the region of the gonad. From three separate transplantation experiments, a total of 322 chimeric larvae were obtained and 30 were found to have DsRed-positive cells in the gonad. The frequency (9%) of potential germ line chimeras was higher than that obtained with cultured zebrafish ES cells (<1%). The DsRed-positive area in the region of the gonad continued to increase in size in the chimeric larvae over a period of two weeks, indicating that the transplanted PGCs were proliferating in the host. Twenty-four of the 30 potential germ line chimeric founders survived to sexual maturity and each was bred with a nonchimeric mate to confirm germ line contribution of the transplanted PGCs. Nine of the chimeric fish transmitted Neo-DsRed sequence to the F1 embryos, indicating that they were germ line chimeras. PCR analysis was conducted on DNA isolated from groups of approximately 50 F1 embryos using primers designed to amplify a Neo-DsRed junction fragment. Thirteen out of a total of 57 batches of embryos (23%) analyzed contained Neo-DsRed sequence. The results revealed that germ line chimeras can be produced from cultured PGCs maintained up to 4 months. Although the inheritance of dnd-Neo-DsRed was detected in F1 embryos by PCR, we did not obtain any larvae that carried the plasmid sequence and continued to survive to sexual maturity. These results suggested that the embryos generated from transplanted PGCs may not be healthy enough to survive, indicating that the cultured PGCs may accumulate genetic aberrations during a long period of time in culture that prevent them from producing normal embryos. To resolve this problem, we are working to identify genetically normal PGC colonies by karyotype analysis as well as to optimize the culture conditions to include other growth factors that have been shown to promote the survival and growth of genetically normal mammalian PGCs. Some of the zebrafish factors that we have cloned and are investigating include growth arrest-specific 6 (Matsubara et al. 1996), gonadal soma-derived growth factor (Sawatari et al. 2007), and LIF (Matsui et al. 1992). Additionally, other germ-cell-specific promoters such as *nanos*, *vasa*, *kop*, and *ziwi* are being used to drive expression of the drug-resistant and fluorescence protein genes in the transgenic embryos. PGC cultures will be isolated from each of these transgenic lines and evaluated for the production of germ-line chimeras.

APPLICATION OF STEM CELL TECHNOLOGY TO MARINE BIOTECHNOLOGY

Application of transgenic strategies to aquatic organisms provides an efficient approach to the genetic improvement of economically important species. The most common method used to generate transgenic fish is by the direct introduction of foreign DNA into fertilized eggs (Chourrout et al. 1986; MacDonald and Ekker, Chapter 15; Fletcher and Davies, Chapter 16). Although this approach is convenient and widely used, it does not provide a means to target the insertion of foreign DNA to a specific site in the fish genome. The development of a stem-cell-mediated gene transfer approach that can be applied to aquatic species will complement standard transgenic strategies currently available and expand the range of genetic manipulations that can be performed on aquaculture species. Recently, targeted deletions were successfully introduced into the zebrafish genome through the use of zinc-finger nucleases (Doyon et al. 2008; Meng et al. 2008). As the use of zinc-finger nucleases for targeted mutagenesis expands, the potential exists to combine this approach with the use of germ line competent fish stem cell lines to introduce targeted knock-ins by homologous recombination using the zinc-finger-nuclease constructs. Although most of the research to date aimed at developing germ-line-competent fish stem cell lines has been done with model species such as zebrafish and medaka, once the technology is developed, the results of these studies will have application to other commercially important aquaculture species. In addition to the production of transgenics, the development of methods to culture fish PGCs will have important applications to the general field of gamete cryopreservation. Currently, the most reliable technique to preserve fish genetic material is by sperm cryopreservation (Scott and Baynes 1980; Herraez et al., Chapter 20). Since the preservation of fish eggs has not been successful, the availability

of eggs from the same species has been a limiting factor in determining if the cryopreserved sperm can be used to reestablish the fish population. The availability of germ-line-competent PGC cell lines could provide an ideal solution to this problem. Using techniques developed by the Yoshizaki et al. (see Chapter 14) for germ cell transplantation into the same or related fish species, the cryopreserved PGC cultures could be introduced into the gonad of recipient fish, where they would differentiate into either eggs or sperm, making it possible to generate viable offspring by in vitro fertilization or natural spawning. This approach would have important implications for reestablishing populations of endangered fish species by providing an inexhaustible supply of germ cells for transplantation.

ACKNOWLEDGMENTS

This work was supported by grants from NIH (R01GM069384) and USDA (2005-35206-15261).

REFERENCES

Bejar J, Hong Y, and Alvarez MC. 1999. Towards obtaining ES cells in the marine fish species *Sparus aurata*: multipassage maintenance, characterization and transfection. Genet Anal. 15: 125–129.

Bejar J, Hong Y, and Alvarez MC. 2002. An ES-like cell line from the marine fish *Sparus aurata*: characterization and chimaera production. Transgenic Res. 11: 279–289.

Braat AK, Zandbergen T, Van De Water S, Goos HJ, and Zivkovic D. 1999. Characterization of zebrafish primordial germ cells: morphology and early distribution of *vasa* RNA. Dev Dyn. 216: 153–167.

Capecchi MR. 1989. Altering the genome by homologous recombination. Science. 244: 1288–1292.

Chen SL, Sha Z, and Ye H. 2003a. Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicus*) embryos. Aquaculture. 218: 141–151.

Chen SL, Ye H, Sha Q, and Xhi CY. 2003b. Derivation of a pluripotnet embryonic cell line from red seabream blastulas. J Fish Biol. 63: 795–805.

Chourrout D, Guyomard R, and Houdebine LM. 1986. High efficiency gene transfer in rainbow trout (*Salmo gairdneri*) by microinjection into egg cytoplasm. Aquaculture. 51: 143–150.

Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, and Smithies O. 1987. Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature. 330: 576–578.

Doitsidou M, Reichman-Fried M, Stebler J, Koprunner M, Dorries J, Meyer D, Esguerra CV, Leung T, and Raz E. 2002. Guidance of primordial germ cell migration by the chemokine SDF-1. Cell. 111: 647–659.

Doyon Y, Mccammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, and Amacher SL. 2008. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol. 26: 702–708.

Durcova-Hills G, Adams IR, Barton SC, Surani MA, and Mclaren A. 2006. The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells. 24: 1441–1449.

Fan L, Crodian J, and Collodi P. 2004a. Culture of embryonic stem cell lines from zebrafish. Methods Cell Biol. 76: 151–160.

Fan L, Crodian J, and Collodi P. 2004b. Production of germ line chimeras for targeted gene disruption. Methods Cell Biol. 77: 113–119.

Fan L, Liu X, Alestrom A, Alestrom P, Crodian J, and Collodi P. 2004c. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish. 1: 22–26.

Fan L, Moon J, Crodian J, and Collodi P. 2006. Homologous recombination in zebrafish ES cells. Transgenic Res. 15: 21–30.

Fan L, Moon J, Wong TT, Crodian J, and Collodi P. 2008. Zebrafish primordial germ cell cultures derived from *vasa::RFP* transgenic embryos. Stem Cells and Development. 17: 585–597.

Ganassin RC and Bols NC. 1999. A stromal cell line from rainbow trout spleen, RTS34ST, that supports the growth of rainbow trout macrophages and produces conditioned medium with mitogenic effects on leukocytes. In Vitro Cell Dev Biol Anim. 35: 80–86.

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, and Daley GQ. 2004. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature. 427: 148–154.

Herpin A, Rohr S, Riedel D, Kluever N, Raz E, and Schartl M. 2007. Specification of primordial germ cells in medaka (*Oryzias latipes*). BMC Dev Biol. 7: 3.

Higashijima S, Okamoto H, Ueno N, Hotta Y, and Eguchi G. 1997. High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev Biol. 192: 289–299.

Hong Y, Winkler C, and Schartl M. 1996. Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). Mech Dev. 60: 33–44.

Hong Y, Winkler C, and Schartl M. 1998a. Efficiency of cell culture derivation from blastula embryos and of chimera formation in the medaka (*Oryzias latipes*) depends on donor genotype and passage number. Dev Genes Evol. 208: 595–602.

Hong Y, Winkler C, and Schartl M. 1998b. Production of medakafish chimeras from a stable embryonic stem cell line. Proc Natl Acad Sci. 95: 3679–3684.

Kawakami K, Amsterdam A, Shimoda N, Becker T, Mugg J, Shima A, and Hopkins N. 2000. Proviral insertions in the zebrafish hagoromo gene, encoding an F-box/WD40-repeat protein, cause stripe pattern anomalies. Curr Biol. 10: 463–466.

Kobayashi T, Takeuchi Y, Takeuchi T, and Yoshizaki G. 2007. Generation of viable fish from cryopreserved primordial germ cells. Mol Reprod Dev. 74: 207–213.

Koprunner M, Thisse C, Thisse B, and Raz E. 2001. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev. 15: 2877–2885.

Krovel AV and Olsen LC. 2002. Expression of a *vas::EGFP* transgene in primordial germ cells of the zebrafish. Mech Dev. 116: 141–150.

Kurita K, Burgess SM, and Sakai N. 2004. Transgenic zebrafish produced by retroviral infection of in vitro-cultured sperm. Proc Natl Acad Sci. 101: 1263–1267.

Kurita K and Sakai N. 2004. Functionally distinctive testicular cell lines of zebrafish to support male germ cell development. Mol Reprod Dev. 67: 430–438.

Liu JP, Baker J, Perkins AS, Robertson EJ, and Efstratiadis A. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell. 75: 59–72.

Ma C, Fan L, Ganassin R, Bols N, and Collodi P. 2001. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc Natl Acad Sci. 98: 2461–2466.

Matsubara N, Takahashi Y, Nishina Y, Mukouyama Y, Yanagisawa M, Watanabe T, Nakano T, Nomura K, Arita H, Nishimune Y, Obinata M, and Matsui Y. 1996. A receptor tyrosine kinase, Sky, and its ligand Gas 6 are expressed in gonads and support primordial germ cell growth or survival in culture. Dev Biol. 180: 499–510.

Matsui Y, Zsebo K, and Hogan BL. 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell. 70: 841–847.

Mellgren EM and Johnson SL. 2005. *kitb*, a second zebrafish ortholog of mouse Kit. Dev Genes Evol. 215: 470–477.

Meng X, Noyes MB, Zhu LJ, Lawson ND, and Wolfe SA. 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol. 26: 695–701.

Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'brien W, Raz E, Littman D, Wylie C, and Lehmann R. 2003. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development. 130: 4279–4286.

Okutsu T, Shikina S, Kanno M, Takeuchi Y, and Yoshizaki G. 2007. Production of trout offspring from triploid salmon parents. Science. 317: 1517.

Parichy DM, Rawls JF, Pratt SJ, Whitfield TT, and Johnson SL. 1999. Zebrafish sparse corresponds to an orthologue of *c-kit* and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. Development. 126: 3425–3436.

Park TS and Han JY. 2000. Derivation and characterization of pluripotent embryonic germ cells in chicken. Mol Reprod Dev. 56: 475–482.

Park TS, Hong YH, Kwon SC, Lim JM, and Han JY. 2003. Birth of germline chimeras by transfer of chicken embryonic germ (EG) cells into recipient embryos. Mol Reprod Dev. 65: 389–395.

Pesce M, Farrace MG, Piacentini M, Dolci S, and De Felici M. 1993. Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development. 118: 1089–1094.

Piedrahita JA, Moore K, Oetama B, Lee CK, Scales N, Ramsoondar J, Bazer FW, and Ott T. 1998. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol Reprod. 58: 1321–1329.

Raz E. 2003. Primordial germ-cell development: the zebrafish perspective. Nat Rev Genet. 4: 690–700.

Raz E. 2004. Guidance of primordial germ cell migration. Curr Opin Cell Biol. 16: 169–173.

Saito T, Goto-Kazeto R, Arai K, and Yamaha E. 2008. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol Reprod. 78: 159–166.

Sawatari E, Shikina S, Takeuchi T, and Yoshizaki G. 2007. A novel transforming growth factor-beta

superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). Dev Biol. 301: 266–275.

Schier AF. 2003. Chemokine signaling: rules of attraction. Curr Biol. 13: R192–R194.

Schulte-Merker S, Ho RK, Herrmann BG, and Nusslein-Volhard C. 1992. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development. 116: 1021–1032.

Schulte-Merker S, Van Eeden FJ, Halpern ME, Kimmel CB, and Nusslein-Volhard C. 1994. No tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. Development. 120: 1009–1015.

Scott AP and Baynes SM. 1980. A review of the biology, handling and storage of salmonid spermatozoa. J Fish Biol. 17: 707–739.

Shikina S, Ihara S, and Yoshizaki G. 2008. Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Mol Reprod Dev. 75: 529–537.

Sun L, Bradford CS, Ghosh C, Collodi P, and Barnes DW. 1995. ES-like cell cultures derived from early zebrafish embryos. Mol Mar Biol Biotechnol. 4: 193–199.

Takeuchi Y, Yoshizaki G, and Takeuchi T. 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod. 69: 1142–1149.

Van De Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, and Etches RJ. 2006. Germline transmission of genetically modified primordial germ cells. Nature. 441: 766–769.

Wakamatsu Y, Ozato K, and Sasado T. 1994. Establishment of a pluripotent cell line derived from a medaka (*Oryzias latipes*) blastula embryo. Mol Mar Biol Biotechnol. 3: 185–191.

Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, and Raz E. 2003. *Dead end*, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol. 13: 1429–1434.

Yoshizaki G, Tago Y, Takeuchi Y, Sawatari E, Kobayashi T, and Takeuchi T. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in salmonidae. Biol Reprod. 73: 88–93.

Zhang W, Behringer RR, and Olson EN. 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes Dev. 9: 1388–1399.

Chapter 13

Culture of Fish Head Kidney Mononuclear Phagocytes and Muscle Satellite Cells: Valuable Models for Aquaculture Biotechnology Research

Frederick W. Goetz, Josep V. Planas, Mònica Díaz, Dimitar B. Iliev, and Simon MacKenzie

INTRODUCTION

In aquaculture-related research, both primary and continuous cell culture systems have provided essential models that have contributed to many important advances in the understanding of basic cellular processes in fish and mollusks, and for the detection of viruses in wild fish populations (Villena 2003). A number of cell lines for commercially important fish species, including salmonids, ictalurids, and acipenserids, are available from the ATCC (American Tissue Culture Collection). In parallel, a number of methodologies have been published, covering the development and use of primary cell cultures derived from a range of tissues, including hepatocytes, brain, gill and skin epithelia, gonads, spleen, blood leukocytes, and head kidney mononuclear phagocytes.

The choice of a particular cell culture system is dependent upon the biological objectives of the research that will be carried out. Continuous cell lines provide several advantages, primarily the capacity for expansion. The relative homogeneity of cell lines also provides an important constant in obtaining biological material required for experimentation. In addition, such cell lines are essential for the development of functional cell-based assays, including transfection-based assays, characterization of promoter function (Ikeuchi et al. 2003), specific gene regulation (Collet et al. 2004), and the use of reporter genes such as luciferase for cytokine bioassays. In contrast, primary culture systems are generally more labor-intensive and require animal holding facilities for a continual supply of tissue. However, a major advantage with primary cell cultures is the retention

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

of the original biological activity of the cells and the integrity of regulatory cellular mechanisms.

MONONUCLEAR PHAGOCYTES IN FISH

Mononuclear phagocytes are a highly diverse group of cells with a pronounced phenotypic plasticity that forms the mononuclear phagocyte system. These cells are found across vertebrates and form a central role in immunity and tissue homeostasis. In mammals, myelopoiesis, the cellular differentiation and development of mononuclear phagocytes, occurs exclusively in the bone marrow where hematopoiesis takes place and hematopoietic stem cells are located. In fish, however, the major hematopoietic organ is the head kidney that has both hematopoietic and endocrine functions (e.g., corticosteroid synthesis). Furthermore, significant anatomical, morphological, and potential functional differences have been observed in this organ across teleosts (Meseguer et al. 1995; Zapata et al. 1996). The relative ease of dissection of this tissue and the subsequent processing (see below) to purify cellular populations has made the head kidney a popular model in fish immunology. This has resulted in a significant number of publications utilizing the head kidney that have provided a wide range of immunological data derived from functional assays such as the phagocytosis of pathogens, cloning and characterization of novel genes, gene expression studies, and microarray analyses. However, the comparison and interpretation of data obtained from these studies is often complex due to the different culture methods that have been employed. Many of the studies have used head kidney cells either directly or after only a small incubation period in vitro and, therefore, the heterogeneity of cell populations and cellular phenotypes present do not represent a homogenous cellular phenotype or response. In contrast, there have been relatively few models developed around the primary cell culture of fish head kidney cells.

Extensive studies on the basic functional properties of the monocytic/macrophage cell phenotype, including adherence to culture plate surfaces, nonspecific esterase activity, chemotaxis, phagocytic activity, differentiation status, and gene expression, have been carried out in goldfish (*Carassius carassius*; Belosevic et al. 2006), turbot (*Scopthalamus maximus*; Tafalla and Novoa 2000), sea bream (*Sparus aurata*; Garcia-Castillo et al. 2002; Donate et al. 2007; Mulero et al. 2008), Atlantic salmon (*Salmo salar*; Skjaeveland et al. 2008), and in diverse trout species (MacKenzie et al. 2003; 2004; Iliev et al. 2005a). These studies have been key in the description of the proinflammatory response in fish to pathogen-associated molecular patterns (PAMPs). In parallel, a number of head kidney and mononuclear-like continuous passage cell lines have been described, including a goldfish macrophage cell line (Neumann et al. 1998) and several trout and salmon lines (ASK, SHK1, RTS11; Brubacher et al. 2000; Neumann et al. 2001; Rolland et al. 2005).

Primary Cell Culture Methods for the Head Kidney

Our initial primary cell culture system for head kidney cells was developed using brook trout (*Salvelinus fontinalis*; MacKenzie et al. 2003), but was later found to be the same using rainbow trout (*Oncorhynchus mykiss*; Goetz et al. 2004). The basic approach for obtaining differentiated macrophages is relatively uncomplicated. Before dissection, the fish is bled as much as possible to reduce bleeding during the removal of the head kidney and to decrease the number of red blood cells in the tissue. After carefully wiping the fish with 70% ethanol, the abdominal cavity is opened from the anus to just below the pericardial cavity, and the gonads, gastrointestinal organs, and liver are retracted or removed. This allows the head kidneys (pronephroi) to be easily observed at the anterior most end of the trunk kidney (Fig. 13.1A). The head kidneys are dissected with sterile instruments and placed in medium in a sterile Petri dish (Fig. 13.1B). The medium used for the isolation of cells and for subsequent incubation is Dulbecco's Modified Eagle Medium (DMEM; Gibco, cat.# 11971-025) containing 4500 mg/L *D*-glucose (high glucose) and *L*-glutamine, but no sodium phosphate or sodium pyruvate. To this medium is added 10% heat-inactivated fetal bovine serum (FBS; e.g., Gibco, cat.# 16140-071) and penicillin (100 units/mL), streptomycin (100 units/mL), and amphotericin B (0.25 ug/mL; antibiotic/fungizone mix–Lonza cat. #17-745H). Under a laminar flow hood, head kidneys are placed between a folded piece of 200 um nylon mesh (Fig.13.1B) and,

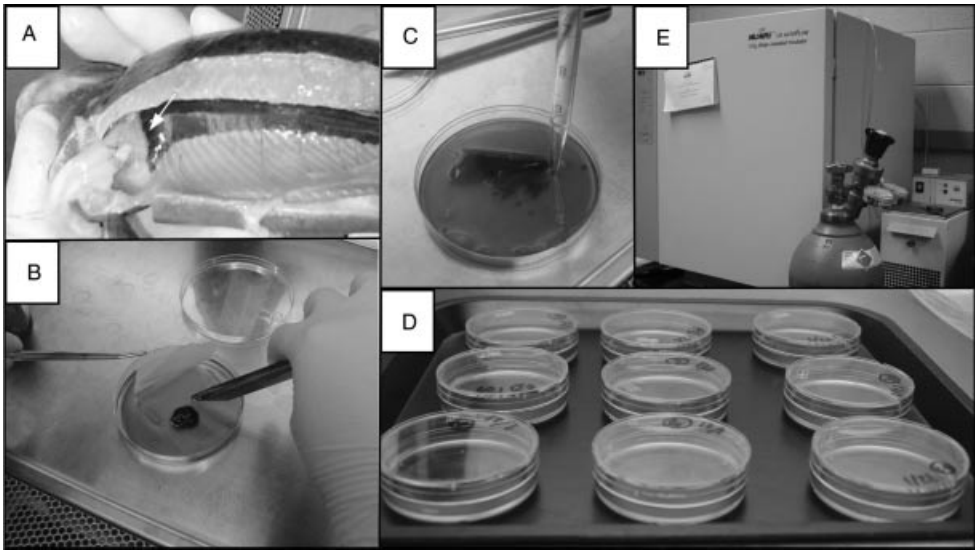


Figure 13.1. Dissection and isolation of head kidney cells from rainbow trout. (**A**) Dissected trout showing long trunk kidney with head kidney at the most anterior end (white arrow). (**B**) Head kidney placed inside of nylon mesh folder. (**C**) Pipetting dispersed cells after squeezing head kidney between nylon mesh. (**D**) Cells plated on 60 mm poly *D*-lysine treated plates. (**E**) Water-jacketed CO_2 incubator with cooling bath shown on right side. (For color detail, please see the color plate section.)

using a spatula, pressure is applied against the top of the mesh to squeeze the head kidney tissue through the nylon mesh. Medium containing dispersed cells is pipetted using the nylon mesh as a sieve (Fig. 13.1C), and new medium is added to the plate and the squeezing process is repeated. Normally, after two repetitions, all of the cells are expelled from the head kidneys, but a smaller amount of medium can be used a third time to wash the plate of any residual cells. All media from the cell dispersions are combined in a sterile falcon tube and can be plated directly or can be further fractionated by straining through 100 and 40 μm nylon filters (Cell Strainer, BD) to remove larger cell aggregates. Additional centrifugation steps, 5 minutes at 1500 rpm, have also been employed, in which the cells from the first purification are recovered as a cell pellet and subsequently resuspended in a desired volume of culture medium. The combination of centrifugation and resuspension has advantages particularly when evaluating different culture media or components of a medium (e.g., increased osmolality for marine species).

Cells are counted in the combined medium using a hemocytometer and then diluted with medium to obtain an appropriate cell concentration for plating (1×10^6 cells/mL or higher). Depending on the experiment or application, cells can be plated on individual plates (60–100 mm diameter; Fig. 13.1D) or on multiwell culture plates. Regardless, plates need to be poly *D*-lysine treated, and precoated plates can be used (Becton Dickinson) or plates can be coated in the laboratory. We normally plate at least 5 mL of a 1×10^6 cells/mL suspension per 60 mm diameter plate, but the exact density can be changed. Plates are incubated at 15°C in a CO_2 incubator at 5% CO_2 (Fig. 13.1E). All of our incubators are water-jacketed CO_2 incubators that were intended for mammalian cell culture (i.e., heated) but are retrofitted by the manufacturer to contain a cooling loop in the water jacket that is connected to an external circulating, refrigerated water bath (e.g., Julabo F200; Fig. 13.1E).

After approximately 24 hours of incubation, plates are gently washed by pipetting the old medium several times across the plate to disperse nonadherent cells so that only adherent cells are retained. The old medium is then removed and an equal volume of new medium replaced. Cells are then incubated for an additional 4 days (5 days total) prior to being used for experiments.

During the incubation process, the head kidney cells that were originally plated and adhered undergo spontaneous differentiation (Fig. 13.2). After 5 days in culture, adherent cells exhibit the phenotypes

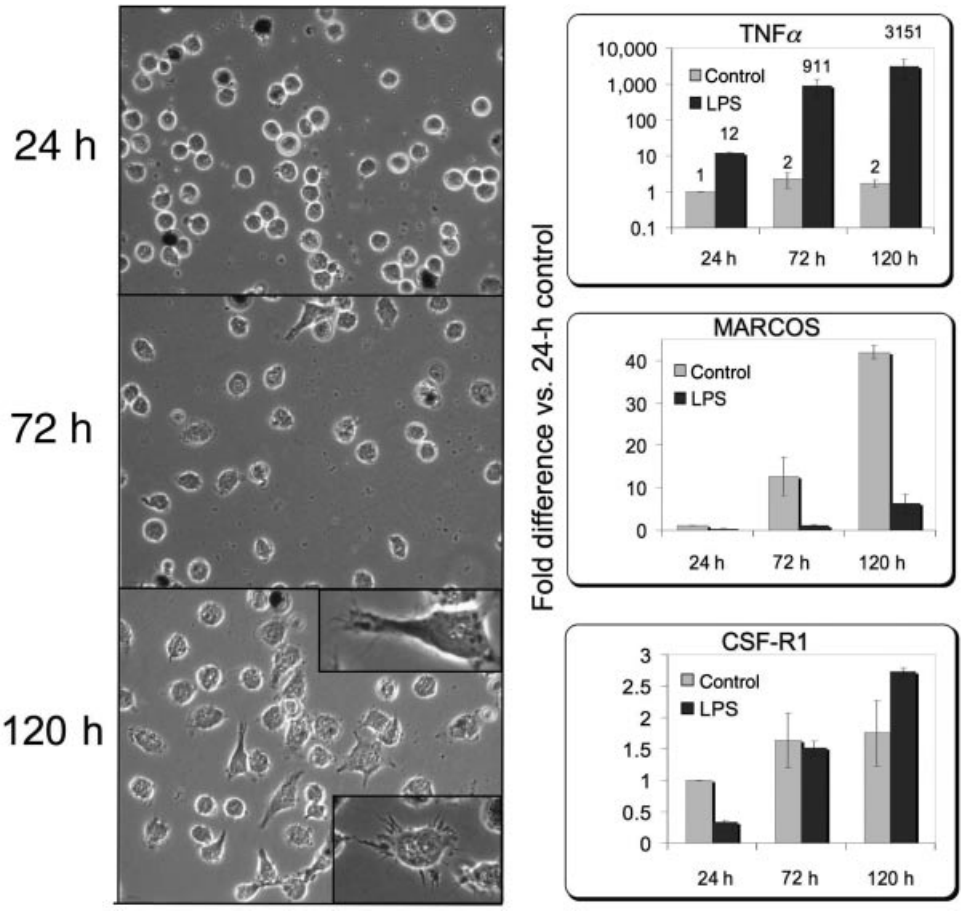


Figure 13.2. *Left*—Microscopic (360×) changes in rainbow trout mononuclear phagocytes undergoing spontaneous differentiation under in vitro conditions. Cells from head kidney homogenates were plated and allowed to adhere for 24 hours after which the nonadherent cells were removed. Inset in bottom panel is high magnification of several differentiated cells. *Right*—Comparative SYBR Green quantitative PCR analysis of the expression of tumor necrosis factor α (TNFα); MARCO; and CSF1R in trout macrophages with or without 25 μg/mL of *E. coli* LPS for 24 h and assayed at 24, 72, or 120 h. Results are shown as the mean of the fold difference of each of the samples vs. the 24 h controls $\pm$ SE ($n = 2$). Note that, the ordinate in panel **A** is logarithmic and the numbers above the histogram columns show the actual fold difference values for the respective samples. (For color detail, please see the color plate section.)

observed in macrophages, including larger and more rounded cells with elongated, branched extensions (Fig. 13.2). We have found that the macrophage lineage markers, macrophage receptor with collagenous structure (MARCO), and colony stimulating factor 1 receptor (CSF1R) are upregulated throughout the culture period (Fig. 13.2). In mammals, MARCO is expressed exclusively in peritoneal macrophages and macrophages that populate lymphoid organs (Elomaa et al. 1995), and its function is implicated in the phagocytosis and clearance of both Gram-negative and Gram-positive bacteria. CSF1R controls the survival, proliferation, and differentiation of macrophages (Roth and Stanley 1992). The presence of CSF1R can be used as a marker for the lineage of the mononuclear phagocytes (Guilbert and Stanley 1980) and its expression, which is controlled at the transcriptional level, increases during the transition from pluripotent hematopoietic cells to monocytes and macrophages (Stanley 1994). Thus, the increased expression of both of these transcripts during incubation supports the hypothesis that the plated head kidney cells are undergoing differentiation to macrophages.

We found that, as the cells differentiate in vitro, their capacity to express cytokines such as tumor necrosis factor α (TNFα), in response to stimulation by PAMPs such as lipopolysaccharide (LPS), increased dramatically (Fig. 13.2). This was also associated with an increase in their ability to phagocytose (MacKenzie et al. 2003; Fig. 13.3). Thus, there is undoubtedly a large amount of transcriptional

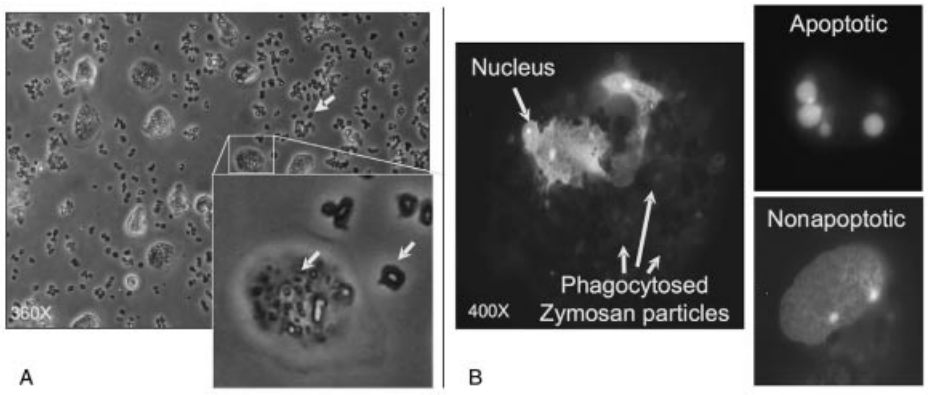


Figure 13.3. Phagocytosis of zymosan by primary trout mononuclear phagocytes. Adherent cells from trout head kidney were cultured for 5 days prior to exposure to zymosan particles. (**A**) Phase contrast imaging of cells exposed to zymosan for 30 h. Zymosan particles are indicated with arrows. (**B**) Fluorescent micrographs of trout macrophages stained with DAPI (a nucleic acid stain). Images include zymosan-exposed and control, nonapoptotic and apoptotic (treated with wortmannin, a PI3 kinase inhibitor) macrophages. Briefly, cells were fixed with 4% paraformaldehyde, washed with PBS, stained, and mounted on microscope slides. Following several washes, zymosan particles remained associated with the cells, indicating that the particles had been internalized. Comparison with the control apoptotic and nonapoptotic cells shows that although the morphology of the nucleus of the phagocytotic cell has been altered, the structure of the chromatin appears to be intact (e.g., it is still composed of clearly discernible heterochromatic and euchromatin regions in contrast with the condensed chromatin of the apoptotic cells). This demonstrates that the cells are highly phagocytic. It also suggests that, following phagocytosis of allogenic material, trout macrophages are able to survive and remain transcriptionally active for a prolonged period, a characteristic of antigen-presenting cells. (For color detail, please see the color plate section.)

activity going on in these cells during the incubation period, providing evidence that they are antigen-presenting cells. While FBS is used in the medium for incubation, we have shown that neither FBS nor homologous trout serum is necessary for the stimulation of proinflammatory cytokine production by LPS in trout macrophages (Iliev et al. 2005a).

We have observed that females that have undergone ovulation (release of oocytes from the follicle), or appear to be in the process of oocyte resorption, have greatly reduced numbers of cells in the head kidney and cannot be used for primary culture (Goetz, unpublished observations). Thus, while large fish appear initially to be good sources for large numbers of head kidney cells, the reproductive status of the fish should be carefully monitored at dissection. The ripening of females can be more of an issue than expected since many commercial trout growers use all-female stocks for enhanced growth and these can mature in 2 years. Slight modifications on the above technique have also been used to develop cultures with similar biological characteristics for other fish species, including cod (*Gadus morhua*; Goetz, unpublished results), yellow perch (*Perca flavescens*; Goetz, unpublished results), gilthead sea bream (Donate et al. 2007), and more recently for turbot and eel (*Anguilla anguilla*; MacKenzie et al., unpublished results). A major factor that should be taken into account in working on different species is that the cellular yield from head kidney tissue can be a limiting factor depending upon the experiments that are desired.

Results of Studies Using Trout Macrophages and Impact on Aquaculture

The primary cell culture of trout macrophages described above has been used for a number of studies looking at gene discovery, expression, and function. Following the initial observation that spontaneously differentiated macrophages produced TNFα under LPS stimulation (MacKenzie et al. 2003), we used the primary cell culture to produce large amounts of mRNA for cDNA library construction aimed at gene discovery (Goetz et al. 2004). Expressed sequence tags of LPS-stimulated trout macrophages yielded a number of interesting immune genes, some of which were subsequently analyzed in relation to expression under various forms of PAMP stimulation. These include the chemokines CCL4 (MacKenzie et al. 2004) and interleukin-6 (IL-6) (Iliev et al. 2006a), the transcription factor PU.1 (Ribas et al. 2008), and components of the respiratory burst process (Boltaña et al. 2009). Later, suppression subtractive hybridization libraries created using trout macrophage cultures stimulated with LPS yielded a number of other PAMP-regulated genes (Iliev et al. 2006b). We have continued to use library-based approaches with trout macrophage cultures to uncover new genes or to evaluate changes in the temporal expression of macrophage genes. For example, in addition to 12-hour LPS-stimulated libraries, we produced cDNA libraries from trout macrophages stimulated with LPS for 30 hours. As stimulation time increased, the overall complexity of the transcripts decreased while the frequency of cytokines such as TNFα, IL-1, and CCL4 increased relative to a 12-hour stimulation (Table 13.1). Interestingly, transcripts for the decoy receptors of TNFα and IL-1 (type II) were more numerous at 30 hours, perhaps in relation to the control/regulation of cytokine increases. However, the most striking difference was that nearly 10% of the transcripts at 30 hours were acute phase serum amyloid A, whereas this transcript only represented about 0.3% of the sequences in the 12-hour library. Serum amyloid A is a protein that is typically produced by the liver under pathogen stimulation (Uhlar and Whitehead 1999).

Using the primary culture system, we have characterized the response (dose and time) of macrophages to a number of PAMPs including LPS, a primary cell well component of Gram-negative bacteria; polyriboinosinic polyribocytidylic acid (poly(I:C)), a synthetic double-stranded RNA viral mimic; and beta-glucan from fungi (Iliev et al. 2005a). During those experiments, it became evident that macrophages responded to phenol-extracted LPS by expressing the transcripts for proinflammatory cytokines such as TNFα and IL-1, but only at concentrations that were 10- to 100-fold greater than the LPS concentrations required to elicit a similar gene response in mammalian macrophages. Subsequently, we showed that the trout macrophages did not respond to ultrapure LPS even at high concentrations (Iliev et al. 2005b). This experimental evidence, together with the in silico analysis of genes involved in the activation of mammalian macrophages by LPS, led us to

Table 13.1. Comparison of Selected EST Frequencies between 12 (1494 ESTs) and 30 (593 ESTs) hour LPS-Stimulated Macrophage cDNA Libraries. See Goetz et al. (2004) for Details on Library Construction and Analysis.

EST Name (based on homology)	EST Size	Accession # of Similar Protein	Species Most Similar to	BLASTx E-value	# of Clones in 30 hour ESTs	# of Clones in 12 hour ESTs
Proteases						
cathepsin K	681	NP_031828	mouse	3e-69	3	0
cathepsin D	818	AAC60301	trout	1e-150	0	19
matrix metalloproteinase 9	813	CAC85923	trout	1e-169	1	16
matrix metalloproteinase	819	BAB19131	trout	1e-122	0	4
Lectins						
galectin-like protein	753	BAA88670	trout	1e-119	8	2
galectin	726	AAF61069	flounder	2e-45	4	0
Cytokines/chemokines						
tumor necrosis factor α	777	CAB92316	trout	1e-106	8	1
TNF decoy receptor	772	AAK91758	trout	1e-151	3	0
interleukin 8	770	CAC33585	trout	5e-41	6	3
interleukin 18	784	CAD89352	trout	1e-111	0	1
interleukin-1β	720	CAA06157	trout	1e-109	17	1
interleukin 1 receptor, type II	757	NP_446405	rat	2e-6	4	2
chemotaxin	718	AAG28030	trout	2e-75	5	0
chemokine CCL4/MIP-1β	808	AAN76071	monkey	5e-12	24	1
natural killer cell enhancement factor	837	AAF71324	trout	1e-113	0	4
Structure/motility						
thymosin B12	683	P26352	trout	0	0	5
Others						
glyceraldehyde 3-phosphate dehydrogenase	869	O42259	trout	1e-153	0	10
heat shock protein 70	791	P08108	trout	1e-146	0	7
differentially regulated trout protein 1	494	AAG30030	trout	2e-45	7	0
acute phase serum amyloid A	690	CAA67766	trout	3e-50	59	5

question the existence of a Toll-like receptor 4-mediated response to LPS in fish (Iliev et al. 2005b). While the mechanism by which LPS stimulates a response in trout is still unknown, these findings have great significance to aquaculture since they may impact the way in which fish are treated for Gram-negative bacterial infections and how fish are treated prophylactically (e.g., using immunostimulant diets). Since the current thought has been that the innate immune response to pathogens is highly conserved within vertebrates, mammalian-based strategies have generally been followed with fish and may not always be effective.

Finally, we have used the trout macrophage culture system with targeted immune microarrays to investigate more global transcriptome responses to PAMP stimulation. Studies on LPS-activation in trout macrophages in primary culture have shown that the overall transcriptomic response, gene circuits, and functional gene ontology classes in trout macrophages are highly similar to those reported in mammals, and that cortisol plays an immunomodulatory role (MacKenzie et al. 2006). Interestingly, a general "LPS-response" group comprising genes involved in the response to external stimuli, stress, immune stimuli, apoptosis, etc. was significantly suppressed with cortisol, whereas other archetypical LPS responses were not modified. It appears, therefore, that the immunomodulatory role of cortisol is more complex than simple antagonism of LPS-induced transcriptional responses (MacKenzie et al. 2006). A highly similar response to LPS was also observed in sea bream macrophages, suggesting that a robust proinflammatory response to bacterial LPS is present in fish separated by large phylogenetic distances. This observation also holds for the antiviral response, studied using poly(I:C) activation in macrophages from both trout and sea bream (MacKenzie et al., unpublished results).

TROUT MUSCLE SATELLITE CELLS

In mammals, it is well accepted that postnatal skeletal muscle growth is dependent on the activation, proliferation, and differentiation of satellite cells, which are muscle stem cells due to their lineage-specific differentiation capability and self-renewal (Buckingham and Montarras 2008). In fish, in contrast to other vertebrates, muscle growth is achieved by hyperplasia and hypertrophy of skeletal muscle fibers and it is believed that hyperplasic growth in fish is the result of the continuous recruitment of satellite cells (Johnston 1999). In view of the central role that satellite cells may play in determining the growth pattern of commercially important fish species, these cells have been the focus of several studies. In particular, during the last 20 years, studies have reported on the isolation and culture of satellite cells in several fish species (e.g., rainbow trout, Atlantic salmon, carp (*Cyprinus carpio*), and zebrafish (*Danio rerio*); Powell et al. 1989; Koumans et al. 1990; Rescan et al. 1995; Fauconneau and Paboeuf 2000), and it is clear that this culture system can provide a useful tool to reproduce the muscle differentiation process in vitro and to study the function and regulation of fish muscle cells. Nevertheless, the question remains as to the exact nature of the cells obtained through the isolation protocols: are these cells true satellite cells or undifferentiated myoblasts? Evidence from Rescan and collaborators (Rescan et al. 1994, 1995) on the rapid expression of differentiation markers such as MyoD and myogenin soon after plating would suggest that these cells may show characteristics of myoblasts. Clearly, further studies are needed to resolve this question.

Primary Cell Culture Methods for Satellite Cells

The most widely used method for the isolation of skeletal muscle cells in fish is that described initially by Fauconneau and Paboeuf (2000). Fish weighing up to 10 g are sacrificed by a blow to the head, immersed in 70% ethanol for 30 seconds in order to sterilize external surfaces, and, after removal of the skin, the dorsal white muscle (approximately 25 g) is isolated in sterile conditions and collected in a 50 mL centrifuge tube with 25 mL of DMEM medium containing 9 mM $NaHCO_3$, 20 mM Hepes, 15% horse serum, and antibiotic–antimycotic cocktail (100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B, 75 mg/mL gentamycin) on ice. After cutting the muscle tissue in small pieces with a scalpel and washing the tissue fragments twice, the tissue is enzymatically digested with a 0.2% collagenase solution in DMEM for 1 hour at 18°C with gentle shaking. The suspension is washed and centrifuged twice (300*g* for 10 minutes at 15°C),

and the resulting pellet is subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 minutes at 18°C and gentle agitation. After each round of trypsinization, the suspension is centrifuged and the supernatant is diluted in 4 volumes of cold DMEM supplemented with 15% horse serum and the same antibiotic–antimycotic cocktail indicated above. After two washes with DMEM, the cellular suspension is filtered through 100 and 40 μm nylon filters (Cell Strainer, BD). Cells are counted and diluted with DMEM containing 10% FBS to reach a final concentration of 4×10^6 cells/mL.

Plastic culture plates (NUNC, Roskilde, Denmark) or coverslips must be treated with poly-*L*-lysine (Sigma, P-6282) and laminin (Sigma, L-2020) to facilitate adhesion and differentiation of satellite cells. First, a solution of poly-*L*-lysine (100 mg/mL, W 70,000–150,000, Sigma) is applied for 5 minutes at 18°C to give a concentration of 4 mg/cm^2. The excess of poly-*L*-lysine is removed and plates are washed with distilled water and air-dried for 1 hour. Secondly, plates and coverslips are incubated with 20 mg/mL laminin for 24 hours at 18°C, and the laminin solution is aspirated just before plating the cell suspension. Cells are cultured on 6-well plates for plasma membrane preparation, RNA extraction, or immunocytochemistry, or 12-well plates for 2-deoxyglucose assays at a density of 3–4 $\times$ 10^6 and 1×10^6 cells/well, respectively. Cells are maintained at 18°C with DMEM containing 9 mM $NaHCO_3$, 20 mM Hepes, 10% FBS, and antibiotic–antimycotic cocktail (100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B). After 24 hours of plating, plates are washed to eliminate those cells not adhered to the well. Medium is routinely renewed every 48 hours. All cultures are morphologically monitored by observation with an inverted microscope.

Once plated on laminin and poly-*L*-lysine substrates, muscle cells from rainbow trout in primary culture undergo morphological changes characteristic of the differentiation process (Fig. 13.4). During the first days of culture, cells are mononucleated with spindle-shape morphology and are named myoblasts. During this period, satellite cells are known to proliferate (Fauconneau and Paboeuf 2001a). On day 4 of culture, cells are more elongated and start to fuse to form small multinucleate cells named myotubes. Subsequently, these small myotubes fuse to

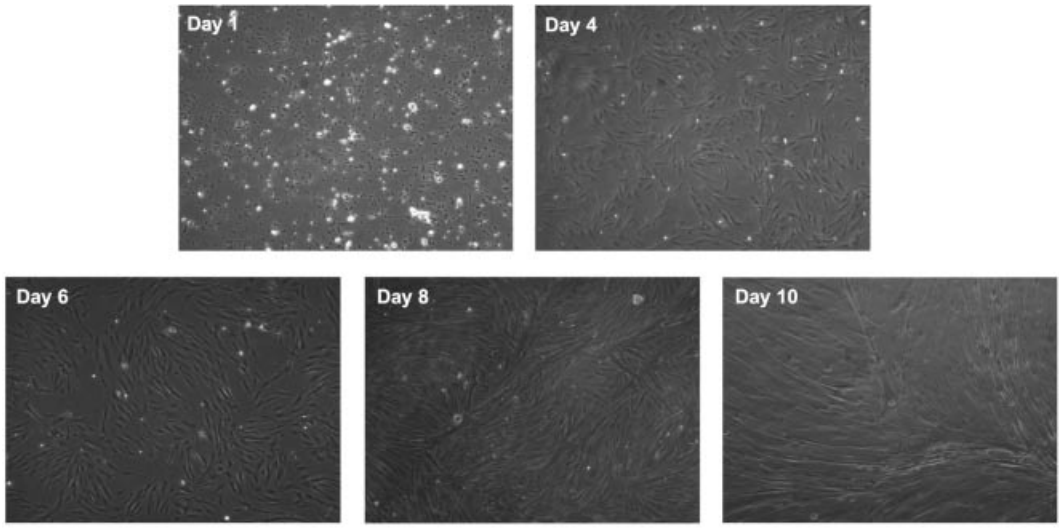


Figure 13.4. Differentiation of rainbow trout muscle satellite cells cultured at 18°C with DMEM containing 10% FBS.

produce large myotubes that can be observed after 8 or 10 days of culture. In mammals, this differentiation process also involves an activation program of muscle-specific genes such as those implicated in muscle contraction, carbohydrate metabolism, or amino acid transport (Moran et al. 2002). During the differentiation of fish muscle cells, the induction of myogenic genes such as myogenin (Rescan et al. 1995) or myosin heavy chain (Gauvry and Fauconneau 1996) has been described.

Functional Characterization of Fish Skeletal Muscle Cells and Their Application in Aquaculture

Muscle satellite cells from rainbow trout have been used as a reporter system for the biological activity of xenobiotic compounds, demonstrating that heavy metals, herbicides, fungicides, and detergents can affect the survival and proliferative capacity of skeletal muscle cells both in vivo and in vitro (Fauconneau and Paboeuf 2001b). Furthermore, trout skeletal muscle cells have been studied in terms of their responsiveness to insulin and IGF-I. First, Castillo et al. (2002) demonstrated the presence of IGF-I and, to a lesser extent, insulin receptors in trout skeletal muscle cells. Second, the uptake of glucose and amino acids by these cells was stimulated by insulin and IGF-I (Castillo et al. 2004).

In our laboratory, we have used the primary culture of skeletal muscle cells as a tool to study the function and regulation of the glucose transporter system in teleosts. As initially shown by Castillo et al. (2002), we have observed stimulatory effects of insulin on glucose uptake in these cells when incubated for periods as short as 5 minutes (Fig. 13.5). The speed at which insulin can stimulate glucose uptake in trout muscle cells in vitro, comparable to that of insulin in mammalian cells, contrasts with the relatively slow regulation of blood plasma levels by insulin in vivo. In order to determine if the stimulation of glucose uptake in trout skeletal muscle cells involved the activation of a hormone-responsive glucose transporter system, we have investigated the expression of GLUT4 at the protein and mRNA levels and its regulation by insulin and IGF-I. By performing immunofluorescence studies using a polyclonal antibody against salmon GLUT4 (Capilla et al. 2004), we have demonstrated that trout skeletal muscle cells express endogenous GLUT4. Trout GLUT4 is localized in intracellular compartments, showing a preferential distribution in the perinuclear region, although GLUT4 immunoreactivity can also be detected in the plasma membrane (Fig. 13.6; Díaz et al. 2007).

The presence of GLUT4 at the plasma membrane in trout muscle cells under basal (nonstimulated) conditions confirms our observations with mammalian cells expressing salmon or trout GLUT4 (3T3-L1 adipocytes and L6 muscle cells, respectively) on the higher percentage of teleost GLUT4 over mammalian GLUT4 at the plasma membrane in the absence of insulin (Capilla et al. 2004; Díaz et al. 2007). This is clearly different from the situation in mammals, since in mammalian muscle cells and adipocytes, most of the GLUT4 is located in intracellular stores in the basal state and is rapidly translocated to the cell surface in response to insulin, resulting in an increase in cellular glucose transport (Watson and Pessin 2006). Our studies on GLUT4 in skeletal muscle cells have allowed us to formulate the hypothesis that the traffic mechanisms governing the appearance of GLUT4 at the cell surface are different between fish and mammals and that intracellular retention of GLUT4 in skeletal muscle cells has improved during the evolution from fish to mammals. These differences in GLUT4 traffic in skeletal muscle cells most likely underlie the well-known differences in glycemic control between fish and mammals.

Using the primary culture of trout muscle cells, we have compared the protein expression of endogenous trout GLUT4 by immunofluorescence between myocytes after 5 days in culture and myotubes after 10 days in culture. In both stages, the localization of trout GLUT4 was mainly intracellular but trout GLUT4 immunoreactivity was more intense in fully differentiated myotubes than in myocytes after day 5 of culture (Díaz et al. 2007). The observation of increased GLUT4 protein content during the transition between myocyte and myotube stages has been corroborated at the mRNA level since a significant increase in the expression of GLUT4 is detected over the course of the myogenic differentiation process (Díaz et al. 2009). On the other hand, the expression of GLUT1, another glucose transporter expressed in the trout skeletal muscle (Capilla et al. 2002), shows

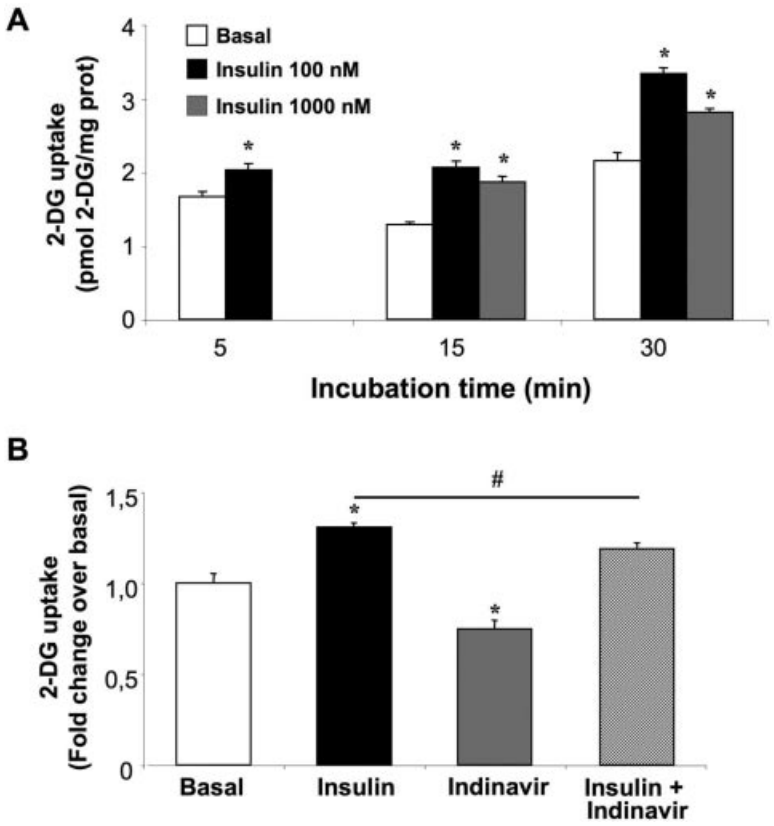


Figure 13.5. (**A**) Time course of insulin-stimulated 2-deoxyglucose (2-DG) uptake in trout muscle cells. After 5 days of culture, cells were serum-starved for 4 h and subsequently incubated for the indicated times with or without insulin (100 or 1000 nM) at 18°C. After the incubation period, a 2-DG uptake assay was performed. Results are expressed as absolute values of pmoles of 2-DG per mg of protein. (**B**) Effect of indinavir on insulin-stimulated 2-DG uptake. After 5 days of culture, cells were serum-starved for 4 h and subsequently incubated in the absence or presence of 1 μM insulin for 30 min at 18°C. After the incubation period, a 2-DG uptake assay was performed. Indinavir was added to the transport solution in a final concentration of 100 μM. Results are expressed as fold stimulation above basal, which was set to 1. Values are mean ± SE from a representative experiment performed in triplicate. *Significant differences compared to basal ($P < 0.05$). # $P < 0.05$.

only a very modest increase during the myogenic differentiation process (Díaz et al. 2009).

To provide evidence for the involvement of GLUT4 in facilitating glucose uptake in trout skeletal muscle cells, we used a selective inhibitor of GLUT4, indinavir (Murata et al. 2002). In trout muscle cells, indinavir caused a significant reduction in both basal and insulin-stimulated glucose uptake (Fig. 13.5), suggesting that GLUT4 is indeed involved, at least in part, in mediating the stimulatory effects of insulin on glucose uptake. In trout muscle cells, indinavir caused a 25% and 9% reduction in basal and insulin-stimulated glucose uptake, respectively, whereas in myotubes from L6 cells, the same concentration of the inhibitor caused about 35% and 50% reduction of both basal and

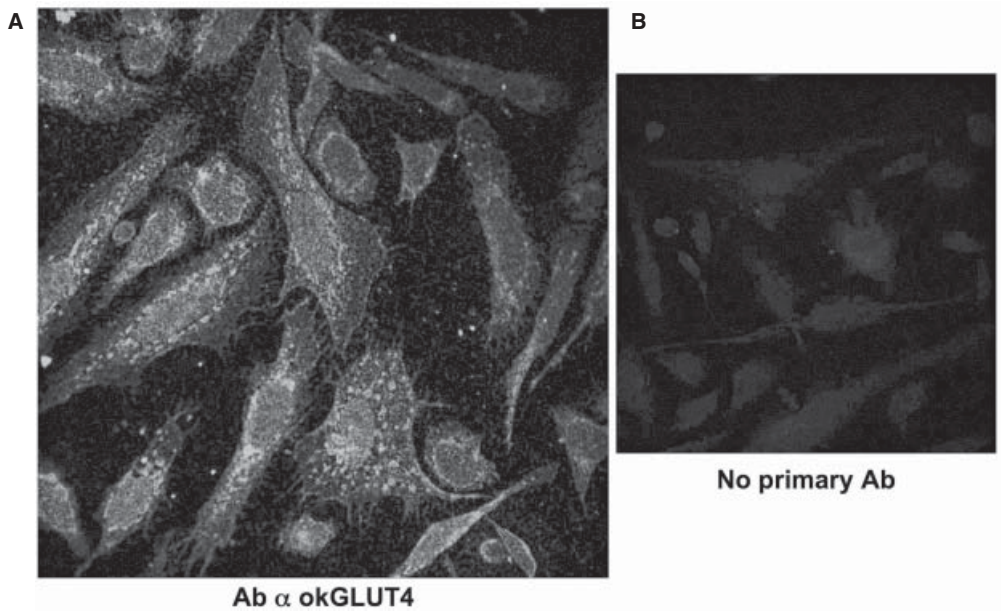


Figure 13.6. Subcellular localization of endogenous GLUT4 in trout muscle cells. (**A**). Immunofluorescence of trout GLUT4 in cells at day 5 of culture using the anti-okGLUT4 (salmon GLUT4) (Capilla et al. 2004) as primary antibody and AlexaFluor488-conjugated antibody. (**B**) Background fluorescence (in the absence of primary antibody), indicating that the staining in Fig. 13.3A was not due to a nonspecific signal from the secondary antibody. (For color detail, please see the color plate section.)

insulin-stimulated glucose transport activity, respectively (Rudich et al. 2003). Therefore, it appears that indinavir is less effective in inhibiting trout GLUT4 than mammalian GLUT4 and we have postulated that this difference in sensitivity could be due to the existing differences between the primary structure of trout and mammalian GLUT4 (Díaz et al. 2007). Furthermore, GLUT4 is not the only GLUT isoform present in trout muscle cells, since these cells also express GLUT1 (Capilla et al. 2002; Díaz et al. 2009). Since we do not know the relative sensitivity of trout GLUT1 to indinavir, it is difficult to conclude from these results what is the relative contribution of trout GLUT4 to glucose transport activity in trout muscle cells. Further investigation will be needed to answer this question.

The trout primary skeletal muscle cell culture system has proved to be instrumental in demonstrating the direct effects of insulin regulating GLUT4 protein and mRNA expression in muscle cells. On one hand, we have shown that insulin rapidly increases the amount of the GLUT4 protein at the plasma membrane of trout muscle cells by stimulating its translocation from intracellular stores (Díaz et al. 2007). This nongenomic effect of insulin is responsible for the increase in glucose uptake in trout skeletal muscle cells (Fig. 13.5). On the other hand, insulin and IGF-I directly stimulate the expression of GLUT4, as well as GLUT1, in trout skeletal muscle cells, similar to what is known in mammals (Díaz et al. 2009). Therefore, our studies on the regulation of GLUT4 in trout skeletal muscle cells clearly indicate that the endocrine mechanisms involved in the rapid translocation of GLUT4 to the plasma membrane (responsible for the increase in glucose uptake), and in the stimulation of the expression of the GLUT4 gene, have been remarkably well conserved between fish and mammals.

Primary cultures of skeletal muscle cells have also been used to study the growth-promoting effects of growth hormone (GH) on skeletal muscle. Levesque et al. (2008) have recently reported that myosatellite cells from Atlantic salmon respond to GH in vitro by increasing their proliferative potential. Furthermore, these authors reported that myosatellite cells isolated from white skeletal muscle from GH-transgenic salmon showed higher proliferative rates than cells isolated from nontransgenic fish. These in vitro results suggest that GH may stimulate somatic growth by increasing the proliferation of skeletal muscle cells.

In conclusion, primary cultures of skeletal muscle cells provide a useful experimental model for studying the effects of factors potentially involved in growth and metabolism as well as for the identification of novel factors that determine these processes. Primary cultures of skeletal muscle cells combined with genomic approaches (microarray analysis, pyrosequencing, proteomic analysis, etc.) may shed light onto the cellular and molecular mechanisms responsible for the hyperplasic and hypertrophic growth characteristics of fish skeletal muscle and assist in devising strategies to increase muscle growth in aquacultured fish.

REFERENCES

Boltaña S, Doñate C, Goetz FW, Mackenzie S, and Balasch JC. 2009. Characterization and expression of NADPH oxidase in LPS-poly(I:C)- and zymosan-stimulated trout (*Oncorhynchus mykiss* W.) macrophages. Fish Shellfish Immunol. 26: 651–661.

Belosevic M, Hanington PC, and Barreda DR. 2006. Development of goldfish macrophages *in vitro*. Fish Shellfish Immunol. 20: 152–171.

Brubacher JL, Secombes CJ, Zou J, and Bols NC. 2000. Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (*Oncorhynchus mykiss*). Develop Comp Immunol. 24: 565–574.

Buckingham M and Montarras D. 2008. Skeletal muscle stem cells. Curr Opin Genetics Dev. 18: 330–336.

Capilla E, Díaz M, Albalat A, Navarro I, Pessin JE, Keller E, and Planas JV. 2004. Functional characterization of an insulin responsive glucose transporter (GLUT4) from fish adipose tissue. Am J Physiol Endocrinol Metab. 287: E348–E357.

Capilla E, Díaz M, Gutierrez J, and Planas JV. 2002. Physiological regulation of the expression of a GLUT4 homolog in fish skeletal muscle. Am J Physiol Endocrinol Metab. 283: E44–E49.

Castillo J, Codina M, Martinez ML, Navarro I, and Gutierrez J. 2004. Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. Am J Physiol Regul Integr Comp Physiol. 286: R935–R941.

Castillo J, Le Bail PY, Paboeuf G, Navarro I, Weil C, Fauconneau B, and Gutierrez J. 2002. IGF-I binding in primary culture of muscle cells of rainbow trout: changes during *in vitro* development. Am J Physiol Regul Integr Comp Physiol. 283: R647–R652.

Collet B, Boudinot P, Benmansour A, and Secombes CJ. 2004. An Mx1 promoter-reporter system to study interferon pathways in rainbow trout. Dev Comp Immunol. 28: 793–801.

Díaz M, Antonescu CN, Capilla E, Klip A, and Planas JV. 2007. Fish glucose transporter (GLUT)-4 differs from rat GLUT4 in its traffic characteristics but can translocate to the cell surface in response to insulin in skeletal muscle cells. Endocrinology. 148: 5248–5257.

Díaz M, Vraskou Y, Gutierrez J, and Planas JV. 2009. Expression of rainbow trout glucose transporters GLUT1 and GLUT4 during *in vitro* muscle cell differentiation and regulation by insulin and IGF-I. Am J Physiol Regul Integr Comp Physiol. 296: R794–R800.

Donate C, Roher N, Balasch JC, Ribas L, Goetz FW, Planas JV, Tort L, and MacKenzie S. 2007. CD83 expression in sea bream macrophages is a marker for the LPS-induced inflammatory response. Fish Shellfish Immunol. 23: 877–885.

Elomaa O, Kangas M, Sahlberg C, Tuukkanen J, Sormunen R, Liakka A, Thesleff I, Kraal G, and Tryggvason K. 1995. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell. 80: 603–609.

Fauconneau B and Paboeuf G. 2000. Effect of fasting and refeeding on *in vitro* muscle cell proliferation in rainbow trout (*Oncorhynchus mykiss*). Cell Tissue Res. 301: 459–463.

Fauconneau B and Paboeuf G. 2001a. Muscle satellite cells in fish. In: Muscle Development and Growth. Fish Physiology. Vol. 18. (ed. Johnston IA). Academic Press, San Diego, CA. pp 73–101.

Fauconneau B and Paboeuf G. 2001b. Sensitivity of muscle satellite cells to pollutants: an *in vitro* and

in vivo comparative approach. Aquat Toxicol. 53: 247–263.

Garcia-Castillo J, Pelegrin P, Mulero V, and Meseguer J. 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics. 54: 200–207.

Gauvry L and Fauconneau B. 1996. Cloning of a trout fast skeletal myosin heavy chain expressed both in embryo and adult muscles and in myotubes neoformed *in vitro*. Comp Biochem Physiol B Biochem Mol Biol. 115: 183–190.

Goetz FW, Iliev D, Liarte C, McKenzie S, Planas J, and McCauley L. 2004. Analysis of genes isolated from lipopolysaccharide stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages. Mol Immunol. 41: 1199–1210.

Guilbert LJ and Stanley ER. 1980. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 85: 153–159.

Ikeuchi T, Kobayashi T, Todo T, and Nagahama Y. 2003. Transgenic cell lines which stably express progestogen receptors (PRS) alpha and beta and the PR-responsive reporter genes. Fish Physiol Biochem. 28: 151.

Iliev D, Liarte C, MacKenzie S, and Goetz FW. 2005a. Activation of rainbow trout (*Oncorhynchus mykiss*) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. Mol Immunol. 42: 1215–1223.

Iliev D, Roach J, MacKenzie S, Planas J, and Goetz FW. 2005b. Endotoxin recognition: in fish or not in fish. FEBS Lett. 579(29): 6519–6528.

Iliev DB, Esteban BC, MacKenzie S, Planas JV, and Goetz FW. 2006a. Cloning and expression analysis of an IL-6 homolog in rainbow trout (*Oncorhynchus mykiss*). Mol Immunol. 44: 1814–1818.

Iliev DB, Goetz G, Mackenzie S, Planas JV, and Goetz FW. 2006b. Pathogen-associated gene expression profiles in trout macrophages. Comp Biochem Physiol Part D Genomic Proteomics. 1: 416–422.

Johnston IA. 1999. Muscle development and growth: potential implications for flesh quality in fish. Aquaculture. 177: 99–115.

Koumans JTM, Akster HA, Dulos GJ, and Osse JWM. 1990. Myosatellite cells of *Cyprinus carpio* (teleostei) *in vitro*—isolation, recognition and differentiation. Cell Tissue Res. 261: 173–181.

Levesque HM, Shears MA, Fletcher GL, and Moon TW. 2008. Myogenesis and muscle metabolism in juvenile Atlantic salmon (*Salmo salar*) transgenic for growth hormone. J Exp Biol. 211: 128–137.

MacKenzie S, Iliev D, Liarte C, Koskinen H, Planas JV, Goetz FW, Mölsä H, Krasnov A, and Tort L. 2006. Transcriptional analysis of LPS-stimulated activation of trout (*O. mykiss*) monocyte/macrophage cells in primary culture treated with cortisol. Mol Immunol. 43(9): 1340–1348.

Mackenzie S, Liarte C, Iliev D, Planas JV, Tort L, and Goetz FW. 2004. Characterization of a highly inducible novel CC chemokine from differentiated rainbow trout (*Oncorhynchus mykiss*) macrophages. Immunogenetics. 56: 611–615.

MacKenzie S, Planas JV, and Goetz FW. 2003. LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and *in vitro* differentiated macrophages. Dev Comp Immunol. 27: 393–400.

Meseguer J, López-Ruiz A, and García-Ayala A. 1995. Reticuloendothelial stroma of the head-kidney from the seawater teleost gilthead seabream (*Sparus aurata L*): an ultrastructural and cytochemical study. Anat Rec. 241: 303–309.

Moran JL, Li Y, Hill AA, Mounts WM, and Miller CP. 2002. Gene expression changes during mouse skeletal myoblast differentiation revealed by transcriptional profiling. Physiol Genomics. 10: 103–111.

Mulero I, Sepulcre MP, Roca FJ, Meseguer J, García-Ayala A, and Mulero V. 2008. Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev Comp Immunol. 32: 1151–1159.

Murata H, Hruz PW, and Mueckler M. 2002. Indinavir inhibits the glucose transporter isoform GLUT4 at physiologic concentrations. Aids. 16: 859–863.

Neumann NF, Barreda D, and Belosevic M. 1998. Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 4: 417–432.

Neumann NF, Stafford JL, Barreda D, Ainsworth AJ, and Belosevic M. 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Dev Comp Immunol. 25: 807–825.

Powell RL, Dodson MV, and Cloud JG. 1989. Cultivation and differentiation of satellite cells from skeletal muscle of the rainbow trout *Salmo gairdneri*. J Exp Zool. 250: 333–338.

Rescan PY, Gauvry L, and Paboeuf G. 1995. A gene with homology to myogenin is expressed in developing myotomal musculature of the rainbow trout and *in vitro* during the conversion of myosatellite cells to myotubes. FEBS Lett. 362: 89–92.

Rescan PY, Gauvry L, Paboeuf G, and Fauconneau B. 1994. Identification of a muscle factor related to MyoD in a fish species. Biochim Biophys Acta. 1218: 202–204.

Ribas L, Roher N, Goetz FW, Iliev D, Tort L, and MacKenzie S. 2008. Characterisation and expression of the transcription factor PU.1 during LPS-induced inflammation in the rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 24(1): 35–45.

Rolland JB, Bouchard D, Coll J, and Winton JR. 2005. Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anemia virus. J Vet Diagn Invest. 17: 151–157.

Roth P and Stanley ER. 1992. The biology of CSF-1 and its receptor. Curr Top Microbiol Immunol. 181: 141–167.

Rudich A, Konrad D, Torok D, Ben-Romano R, Huang C, Niu W, Garg RR, Wijesekara N, Germinario RJ, Bilan PJ, and Klip A. 2003. Indinavir uncovers different contributions of GLUT4 and GLUT1 towards glucose uptake in muscle and fat cells and tissues. Diabetologia. 46: 649–658.

Skjaeveland I, Iliev DB, Zou J, Jorgensen T, and Jorgensen JB. 2008. A TLR9 homolog that is up-regulated by IFN-gamma in Atlantic salmon (*Salmo salar*). Dev Comp Immunol. 32: 603–607.

Stanley ER. 1994. Colony stimulating factor-1 (macrophage colony stimulating factor). In: The Cytokine Handbook (ed. Thompson A). 2nd ed. Academic Press, London. pp 387–418.

Tafalla C and Novoa B. 2000. Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. Dev Comp Immunol. 24: 623–631.

Uhlar CM and Whitehead AS. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 265(2): 501–523.

Villena AJ. 2003. Applications and needs of fish and shellfish cell culture for disease control in aquaculture. Rev Fish Biol Fish. 13: 111–140.

Watson RT and Pessin JE. 2006. Bridging the GAP between insulin signaling and GLUT4 translocation. Trends Biochem Sci. 31: 215–222.

Zapata AG, Chibá A, and Varas A. 1996. Cells and tissues of the immune system of fish. In: The Fish Immune System: Organism, Pathogen and Environment (eds. Iwama G and Nakanishi T). Academic Press, London. pp 1–62.

Chapter 14

Germ Cell Transplantation in Fish: Basic Biology and Biotechnological Applications

Goro Yoshizaki, Tomoyuki Okutsu, and Yutaka Takeuchi

INTRODUCTION

We induced masu salmon (*Oncorhynchus masou*) to produce rainbow trout (*Oncorhynchus mykiss*) eggs and sperm that developed into normal trout. This is the first experimental case in which healthy fish offspring of one species have been obtained from eggs and sperm produced in another different species. This technology has various promising applications. For example, the bluefin tuna (*Thunnus thynnus*) takes 3–5 years to reach sexual maturity, which is difficult to achieve in captivity. Moreover, the body weight of mature bluefin tuna can reach several hundred kilograms. As a result, seed production for this species is expensive in terms of time, cost, labor, and space. However, if bluefin tuna spermatogonia could be transplanted into the mackerel (*Scomber japonicus*), a closely related species that reaches maturity in just 1–2 years at a body weight of ∼500 g, bluefin tuna gametes might be more easily and rapidly produced, even in a small fish tank. This is particularly useful when we use this system for stock enhancement programs by releasing bluefin tuna juveniles. Another possibility is to produce the eggs and sperm of species such as sturgeon, which require 10 years or more to mature, in closely related fish species that mature in a shorter time period.

As shown in Fig. 14.1, the principle for this technology is very simple. Progenitors of germ cell lineages are isolated from the target species and transplanted into the recipient species. Donor-derived eggs and sperm are then produced in the recipient. However, to make this process successful, it is necessary to combine the use of several key biological properties of living organisms. In the present article, we introduce these properties while outlining how we generated masu salmon to produce 100% rainbow trout offspring.

VASA GENE IS SPECIFICALLY EXPRESSED IN GERM CELLS

In order to produce eggs and sperm of rainbow trout in masu salmon, we first thought of transplanting the progenitor cells of rainbow trout eggs and sperm into masu salmon. It is known that females make eggs from oogonia through oocytes and that males make sperm from spermatogonia through spermatocytes. If we go back to an earlier developmental stage, we arrive at the primordial germ cells that are

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

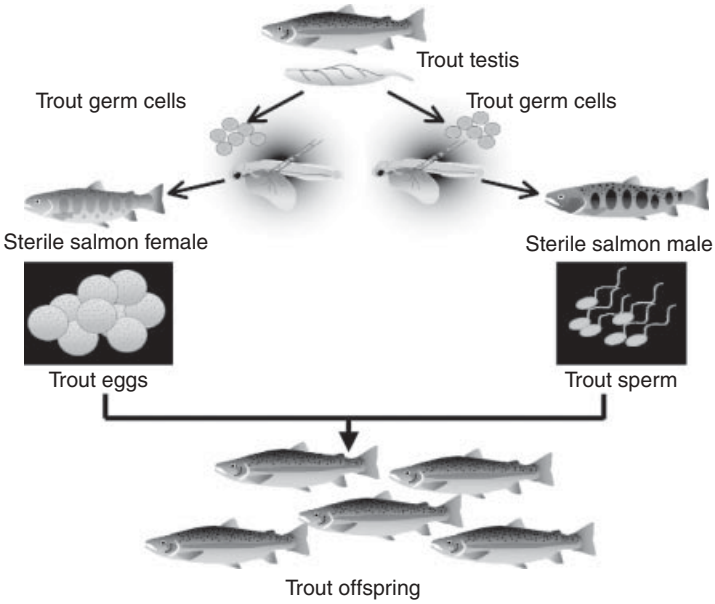


Figure 14.1. Principle for production of eggs and sperm of a different species in surrogate parents by germ cell transplantation. (For color detail, please see the color plate section.)

present in hatching-stage embryos. Primordial germ cells in lower vertebrates, including fish, are sexually undifferentiated and, therefore, have the capacity to differentiate into either eggs or sperm (Devlin and Nagahama 2002). Therefore, we reasoned that if we transplanted rainbow trout primordial germ cells into masu salmon, these cells may produce rainbow trout eggs and sperm.

We first conducted studies with the aim of isolating living primordial germ cells from newly hatched rainbow trout embryos. When we started this research, the technology to isolate living primordial germ cells had not been developed. We therefore focused on the vasa gene, which is specifically expressed in germ cells including primordial germ cells, in a wide range of animal species from *Drosophila* to humans (Raz 2000). In fact, the results of expression analysis after cloning the vasa gene cDNA from rainbow trout showed that this gene is specifically expressed in primordial germ cells of hatching stage embryos (Yoshizaki et al. 2000a). We then produced transgenic trout in order to identify living primordial germ cells.

We prepared plasmids that linked *cis*-regulatory elements of the rainbow trout vasa gene to green fluorescent protein (GFP) genes derived from *Aequorea victoria* and microinjected them into fertilized eggs of rainbow trout (Yoshizaki et al. 2000b). By creating a stable line of these transgenic rainbow trout, we succeeded in producing trout in which both primordial germ cells and spermatogonia in males and primordial germ cells, oogonia, and oocytes in females that could be specifically visualized with green fluorescence (Fig. 14.2; Yoshizaki et al. 2000b; Takeuchi et al. 2002). Using the green fluorescence emitted by these cells as a marker, we were able to purify living primordial germ cells from rainbow trout embryos using a flow cytometer (Takeuchi et al. 2002; Kobayashi et al. 2004). As described above, we succeeded in obtaining living primordial germ cells by using the specific expression of the vasa gene. The next question was how to transplant these cells into recipient individuals.

NEWLY HATCHED EMBRYOS ARE IMMUNOLOGICALLY IMMATURE

When transplanting cells from one species to another, the first problem faced is rejection of the transplanted cells by the immune system of the recipient. This is not limited to the transplantation of germ

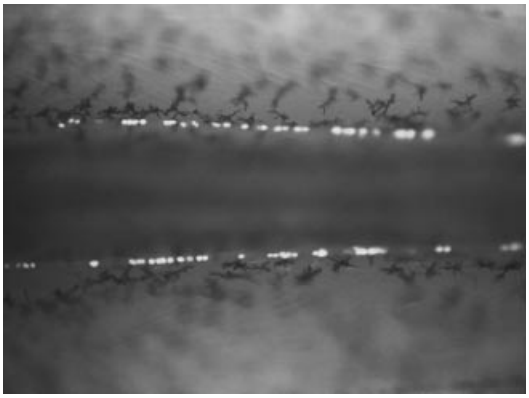


Figure 14.2. Primordial germ cells of rainbow trout transfected with the vasa-GFP gene. Photograph of the ventral side of the peritoneal cavity. The fluorescent cells aligned in two rows along the dorsal wall are primordial germ cells. (For color detail, please see the color plate section.)

cells between species; it is a difficult problem that always needs to be addressed in many other cases such as allogeneic human organ transplantation.

In order to overcome this issue, we focused on the immune system ontogeny of individual animals. Irrespective of animal species, the immune system is undeveloped during the early stages of growth. As an extreme example, no rejection occurs in mammalian blastocysts transferred between species, making it possible to make chimeric individuals by cell transplantation (Fehilly et al. 1984). More interestingly, it is known that in many animal species, the period during which thymus cells or T cells remain undifferentiated continues for some time during embryonic development. In fact, it has long been known that immune immaturity in salmonid fish lasts for about 2 weeks after hatching, during which time they do not have the capacity to reject foreign proteins (Manning and Nakanishi 1996). Therefore, we aimed to exploit this phenomenon to avoid rejection of transplanted germ cells.

We predicted that if we transplanted rainbow trout primordial germ cells into newly hatched masu salmon, they would not be rejected. However, every time an experiment like this is initiated, there are always problems to overcome. Newly hatched masu salmon are small, measuring about 1.3 cm, and their gonads are extremely small. Therefore, the operation of transplanting cells into the recipient gonads was impossible even with the use of a micromanipulator under a microscope. We thus focused on a truly unique ability of primordial germ cells themselves.

GERM CELLS CAN SEEK OUT THE GONADS AND MIGRATE TO THEM

Gonads are made up of germ cells and the somatic cells that surround them. Immature gonads include sexually undifferentiated primordial germ cells that will differentiate into oogonia or spermatogonia as sex differentiation occurs (Patiño and Takashima 1995). The survival proliferation and differentiation of these germ cells are supported and regulated by the surrounding somatic cells. It is very interesting that during this growth process all animals first form the gonadal anlagen that only consists of somatic cells. Primordial germ cells differentiate outside of the gonads and then seek out the location of the gonadal anlagen and migrate there using pseudopodia (Yoshizaki et al. 2002). In other words, the gonads are first built like empty houses, and the primordial germ cells that will become "residents" seek out and migrate to these locations under their own power.

As we were unable to transplant the rainbow trout primordial germ cells directly into the gonads of the masu salmon, we hypothesized that if we transplanted them in the vicinity of gonads, they would migrate toward them under their own power and be incorporated into the gonadal anlagen. We attempted transplanting primordial germ cells into various locations in the newly hatched recipient embryos and eventually discovered that simply transplanting them into the peritoneal cavity (Fig. 14.3) enabled them to migrate to and be incorporated into the recipient gonads where they proliferated and differentiated (Takeuchi et al. 2003).

Thirty-seven male recipient salmon grew to sexual maturity within a year following germ cell injection. Milt produced by these individuals was analyzed by polymerase chain reaction (PCR) to determine the presence of trout-derived sperm. As mentioned above, the donor trout were transgenic

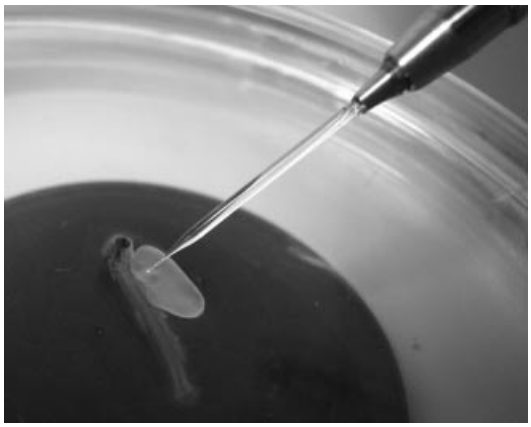


Figure 14.3. Microinjection of germ cells into the peritoneal cavity of newly hatched embryos. (For color detail, please see the color plate section.)

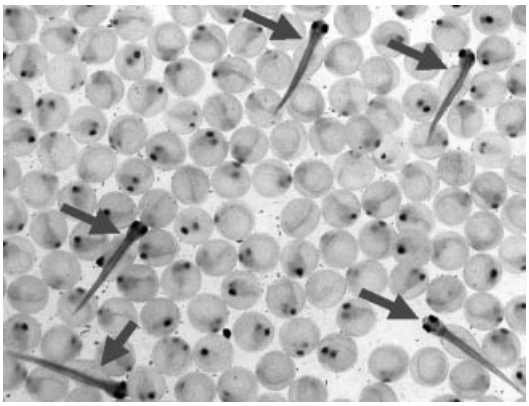


Figure 14.4. Rainbow trout embryos (alevins) obtained from male masu salmon parents following transplantation of trout primordial germ cells (indicated by arrows). The prehatch embryos are hybrids between trout and salmon.

for a vasa-GFP gene. Therefore, PCR analysis using primers specific for the GFP gene made it possible to determine whether milt from the recipient salmon contained trout sperm. The results indicated that 5 of these 37 salmon were producing rainbow trout sperm.

Subsequently, we conducted progeny tests by fertilizing normal rainbow trout eggs with milt from the masu salmon recipients. We predicted that even though the milt from these five individuals contained trout sperm, it would also contain large amounts of masu salmon sperm. Therefore, hybrids would be created if rainbow trout eggs were fertilized by the masu salmon sperm that was contained within the milt. However, we anticipated that hatching of these hybrids would be delayed and that the embryos would die. On the other hand, if the normal trout eggs were fertilized by the trout sperm from the recipient salmon, we predicted that perfectly normal rainbow trout embryos would be produced. Figure 14.4 shows a photograph of offspring individuals. Although many of the individuals were hybrids as evidenced by their delayed hatching, the fry (indicated by the arrows in Fig. 14.4) hatched with exactly the same timing as normal rainbow trout. We judged these individuals to be offspring produced by the fertilization of a rainbow trout egg by a rainbow trout sperm that originated from the transplanted rainbow trout primordial germ cells.

A detailed analysis of these newly hatched embryos revealed the presence of the vasa-GFP genes that were introduced into the donor trout, and that their primordial germ cells emitted green fluorescence. The DNA of these embryos was then analyzed by random amplified polymorphic DNA-PCR (RAPD-PCR), and the resulting banding patterns were found to match perfectly with that of normal rainbow trout. We concluded from this evidence that rainbow trout sperm produced by the masu salmon originated from the transplanted trout primordial germ cells (Takeuchi et al. 2004).

Despite the preceding success, two problematic issues remained. The first was that all of the recipient salmon were males that produced trout-derived sperm. None of the recipients produced trout-derived eggs. The second problem was that the trout sperm produced by the salmon accounted for no more than 0.4% of the sperm, and the remaining 99.6% were from masu salmon. In order to overcome these problems, we experimented with the use of spermatogonia.

TRANSDIFFERENTIATION OF SPERMATOGONIA FROM ADULT FISH INTO EGGS

One problem with conducting primordial germ cell transplantation experiments is the difficulty involved in isolating them. These cells are only found in hatching-stage embryos in very low abundance (about 50–100 cells), and the number of primordial germ cells that can be isolated through enzymatic treatment is only 20–30 per fish. In the above transplant experiments, we found that we needed large numbers of hatched embryos in order to transplant 10–20 primordial germ cells into a single recipient. If this technique were to be applied to commercially valuable species, such as bluefin tuna, the collection of large numbers of embryos having primordial germ cells would be much more difficult than collecting primordial germ cells from rainbow trout. Thus, we focused on the spermatogonia that are present in considerably greater abundance in the testes of male individuals regardless of their age and reproductive state.

Studies on mammals have shown that there are cell populations among spermatogonia that have the capacity to act as stem cells, that is cells that have a combination of self-renewal and differentiation abilities (Yoshida et al. 2007). This led us to isolate spermatogonia from rainbow trout, with the anticipation that they contained spermatogonial stem cells, and transplant them into masu salmon recipients. In this study, we used vasa-GFP transgenic rainbow trout knowing that the vasa gene is also expressed in spermatogonia. As the vasa-GFP gene is expressed in spermatogonia of male adult fish (Yano et al. 2008), we used GFP fluorescence as a marker to purify them by flow cytometery. Cell populations obtained using this method were then transplanted into newly hatched rainbow trout embryos (allogeneic transplantation).

We found that spermatogonia prepared from adult fish and transplanted into the peritoneal cavity of sexually undifferentiated embryos immediately after hatching migrated to and entered the recipient gonads. If the recipient salmon developed into a male, the donor-derived spermatogonia matured into spermatocytes and subsequently to sperm. If the recipient developed into a female, the spermatogonia were taken up in the ovaries of the recipient, and then, through the stages of oogonia and oocytes, differentiated into mature eggs. Thus, it was demonstrated that spermatogonia from adult fish testes, in coordination with embryonic somatic cells in the gonads, can undergo spermatogenesis or oogenesis depending on the gender of the recipient salmon (Okutsu et al. 2006a). Further experiments demonstrated that cross breeding the donor-derived sperm and donor-derived eggs with wild-type eggs and sperm produced normal rainbow trout (Okutsu et al. 2006a).

These results indicate that spermatogonia, which were traditionally thought to only have the capacity to differentiate into sperm, also have the capacity to differentiate into eggs. Moreover, this sexual plasticity of spermatogonia is not only present in individuals immediately following sexual differentiation, it also persists in adult fish that have already matured (Okutsu et al. 2006a). This discovery was a major breakthrough in terms of the practical application of germ cell transplantation. The fact that spermatogonia transplanted into newly hatched embryos can differentiate into either sperm or eggs depending on the sex of the recipient means that they can be used in place of the primordial germ cells. Unlike primordial germ cells, large quantities of spermatogonia can be obtained from a single male fish. This contributes significantly to making these experiments more universally applicable.

On the basis of these findings, we carried out experiments to transplant rainbow trout spermatogonia into masu salmon. In contrast to the small number of primordial germ cells (<50) that were available for transplantation, spermatogonia could be isolated and injected into the peritoneum in large numbers ($>10{,}000$). By simply increasing the number of transplanted cells, we anticipated an increase in transplantation efficiency in terms of the production of donor-derived fish and the successful production of donor-derived rainbow trout eggs.

Thirty-three male masu salmon recipients matured by the spawning season of year 2 following transplantation. PCR analysis of DNA extracted from the milt obtained from these fish using GFP primers revealed that 16 (48%) of salmon were producing trout-derived sperm (Okutsu et al. 2008). The milt from nine of these salmon (56%) produced

normal GFP-positive rainbow trout (F1 generation) when crossed with wild-type trout eggs. Although the milt from the remaining seven salmon was GFP-positive, it is clear that the proportion of sperm carrying this gene was too small to show up in the progeny. The mean percentage of donor-derived trout produced from the aforementioned nine salmon in the F1 generation was 18.9%. This result is considerably better than that obtained using primordial germ cells (0.4%)

Thirty-eight female salmon spermatogonia recipients also matured to produce eggs. When these eggs were fertilized by milt from wild-type rainbow trout milt, one of them produced two donor-derived trout (Fig. 14.5).

These results demonstrate that transplanting large numbers of the abundantly available spermatogonia into masu salmon was much more efficient than transplanting low numbers of primordial germ cells in the production of donor-derived male rainbow trout (18.9% vs. 0.4%).

In addition, the successful production of functional rainbow trout eggs by female masu salmon recipients was demonstrated for the first time (Okutsu et al. 2008).

The use of spermatogonia was a major breakthrough in our series of studies to produce rainbow trout from surrogate masu salmon. However, a remaining issue that needed to be resolved was the fact that the F1 generation produced by the recipients included not only donor-derived individuals, but also large numbers of individuals originating from the gametes produced by the recipient itself. We next attempted to generate masu salmon that only produce rainbow trout gametes.

TRIPLOID FISH ARE STERILE, BUT THEIR SOMATIC CELLS ARE NORMAL

In order to ensure that the surrogate masu salmon could not produce their own gametes, they would need to be sterile. One effective method used to produce sterile salmon is to render them triploid (Thorgaard et al. 1992). Triploid masu salmon, both males and females, have been found to be completely sterile (Yoshizaki et al., unpublished data). Therefore, experiments were conducted to transplant spermatogonia derived from vasa-GFP transgenic rainbow trout into triploid recipient salmon. A histological examination of the testes of two-year-old male recipients immediately before the spawning season revealed that they were full of mature sperm, while testes from control triploid salmon only contained undifferentiated spermatogonia (Fig. 14.6). During the spawning season, 10 of the 29 triploid males examined showed normal secondary sexual characteristics and produced milt. It was impossible to collect any milt from the normal triploid individuals that had not received trout cells (Okutsu et al. 2007).

The ovaries of 17-month-old female recipient triploid salmon contained vitellogenic oocytes that were identical to those seen in normal diploid salmon (Fig. 14.7). Green fluorescence was clearly emitted

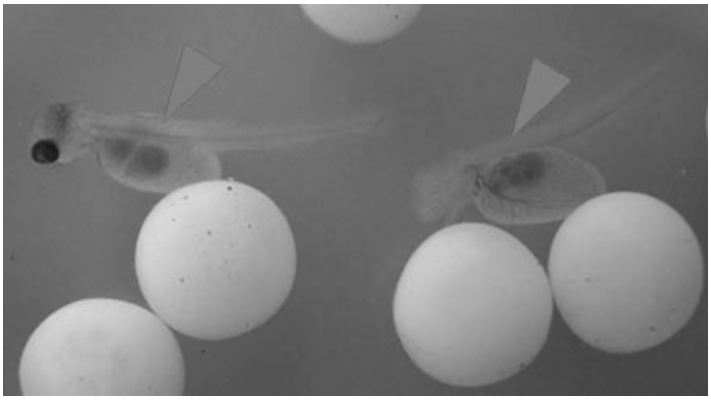


Figure 14.5. Rainbow trout embryos obtained from female masu salmon parents following transplantation of rainbow trout spermatogonia (arrows). The opaque (dead) eggs are hybrids between trout and salmon.

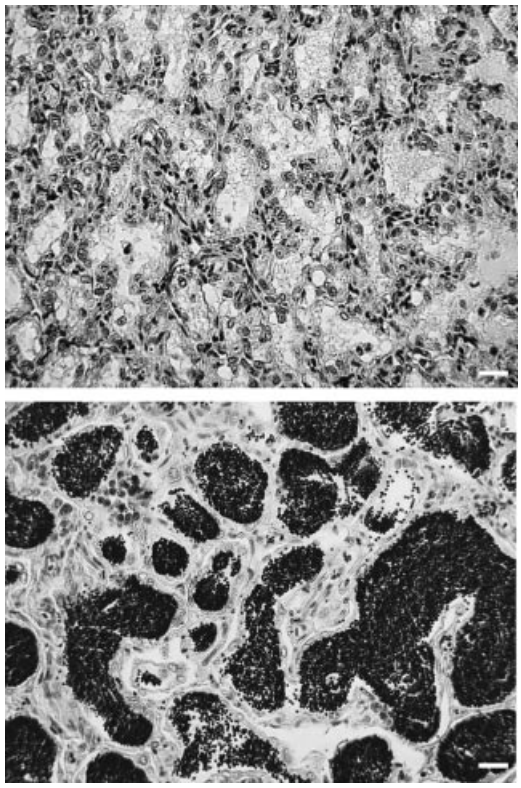


Figure 14.6. Cross sections of testis triploid masu salmon. Upper panel is of a normal triploid salmon. No sperm are present. Lower panel is of a recipient triploid salmon into which rainbow trout spermatogonia had been transplanted. Scale, 20 μm. (For color detail, please see the color plate section.)

by all of the vitellogenic oocytes in the germ cell recipients, indicating that these oocytes were derived from rainbow trout. When the recipients were 2–3 years of age, 5 of 50 female triploid recipients examined ovulated. There was, of course, no ovulation in the normal triploid individuals, and vitellogenesis was not seen.

All offspring generated from mating crosses between sperm and eggs from these mature triploid recipient salmon and the germ cells of wild-type rainbow trout developed normally (Fig. 14.8) and carried the GFP donor trout marker gene. This indicates that all of the F1 generation were produced by donor derived eggs and sperm. This was confirmed through RAPD-PCR and RFLP that the nuclear and mitochondrial genomes of these newly hatched embryos were identical to that of normal rainbow trout. These rainbow trout embryos born from masu salmon parents also grew normally, and in the spawning season of the following year were able to produce normal F2 offspring (Okutsu et al. 2007).

As described above, we have succeeded in markedly increasing the production efficiency of donor-derived offspring from germ cell transplantation by using spermatogonia as donor cells and sterile triploid recipients. The reason that triploids are sterile is thought to be failure of germ cell meiosis. However, questions remained as to whether gonad somatic cells of triploids could nurture normal diploid germ cells and whether the fish's reproductive endocrine system could function normally. The present study clearly demonstrates that the sterility of triploid fish is due entirely to germ cell malfunction and that all other systems associated with reproduction are normal.

CONCLUSION AND PERSPECTIVES

We succeeded in creating masu salmon that produce 100% rainbow trout offspring. The results also indicate that an identical outcome can be obtained without using GFP-marked germ cells.

These results demonstrate that surrogate parent fish of one species can be used to produce offspring of other different species and paves the way for future research on the production of aquaculture species such as the bluefin tuna using mackerel as surrogate parents (Okutsu et al. 2006b). Furthermore, this surrogate parent technology is not only useful for aquaculture but also expected to be useful in preserving the genetic resources and diversity of fish. When an animal species is facing the threat of extinction, the first measure that should be taken is, without question, to protect and restore its native habitat. However, the restoration of aquatic environments is predicted to require a very long time. In some cases, species at risk may become extinct before their habitats are restored. Cryopreservation technologies being developed for sperm, eggs, and embryos are considered to be a viable safety net that could take care of such an event. However, cryopreservation of fish eggs and embryos has not

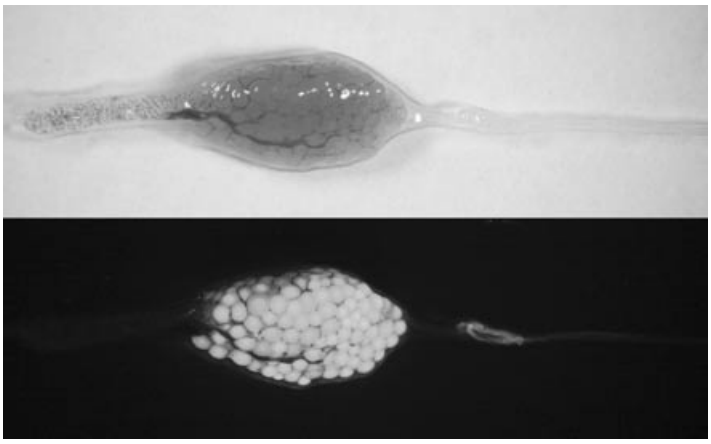


Figure 14.7. Ovaries of triploid masu salmon recipients into which rainbow trout spermatogonia had been transplanted. The top is a bright field image, and the bottom is a fluorescent field image. (For color detail, please see the color plate section.)

been successful to date (Chapter 20 of this book by Herráez et al.).

If primordial germ cells or spermatogonia, which can differentiate into both eggs and sperm, could be cryopreserved, it may be possible to restore extinct species by transplanting frozen cells into closely related living species to produce eggs and sperm of the extinct species, and then to fertilize those eggs and sperm (Kobayashi et al. 2007). We have already started a project to cryopreserve spermatogonia of salmonid fish that are in danger of extinction, and in the future, we plan to build a germ cell bank for fish similar to seed banks for plants (Okutsu et al. 2007).

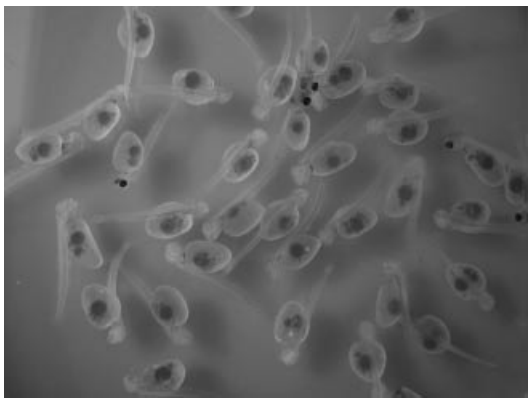


Figure 14.8. Rainbow trout embryos (alevins) obtained by fertilization of an egg and sperm from triploid masu salmon into which rainbow trout spermatogonia had been transplanted.

In this article, we introduced new developmental biotechnology techniques using fish germ cell transplantation. As the reader may have noticed, in this line of experiments, we have done nothing more than to combine certain biological characteristics of living organisms to build an effective method: (i) the vasa gene is specifically expressed in the germ cells of all animals, (ii) newly hatched fish embryos are immunologically immature, (iii) germ cells can seek out and migrate to the gonads, (iv) fish spermatogonia differentiate into eggs as well as sperm, and (v) although triploid fish are sterile, their somatic cells are normal and capable of nurturing diploid germ cells. We would like to further refine these techniques in the future and to actually create mackerel that produce tuna. We will also attempt to produce eggs and sperm from germ cells that have been cultured in vitro over long periods of time.

REFERENCES

Devlin R and Nagahama Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. 208: 191–364.

Fehilly CB, Willadsen SM, and Tucker EM. 1984. Interspecific chimaerism between sheep and goat. Nature. 307: 634–636.

Kobayashi T, Takeuchi Y, Takeuchi T, and Yoshizaki G. 2007. Generation of viable fish from cryopreserved primordial germ cells. Mol Reprod Dev. 74: 207–213.

Kobayashi T, Yoshizaki G, Takeuchi Y, and Takeuchi T. 2004. Isolation of highly pure and viable primordial germ cells from rainbow trout by GFP-dependent flow cytometry. Mol Reprod Dev. 67: 91–100.

Manning MJ and Nakanishi T. 1996. The specific immune system: cellular defences. In: The Fish Immune System (eds. Iwama G and Nakanishi T). Academic Press, New York. pp 159–205.

Okutsu T, Shikina S, Kanno M, Takeuchi Y, and Yoshizaki G. 2007. Production of trout offspring from triploid salmon parents. Science. 317: 1517.

Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, and Yoshizaki G. 2006a. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci USA. 103: 2725–2729.

Okutsu T, Takeuchi Y, and Yoshizaki G. 2008. Spermatogonial transplantation in fish: production of trout offspring from salmon parents. In: Fisheries for Global Welfare and Environment (eds. Tsukamoto K, Kawamura T, Takeuchi T, Beard TD, and Kaiser M). Terrapub, Tokyo. pp 209–219.

Okutsu T, Yano A, Nagasawa K, Shikina S, Kobayashi T, Takeuchi Y, and Yoshizaki G. 2006b. Manipulation of fish germ cells: visualization, cryopreservation and transplantation. J Reprod Dev. 52: 685–693.

Patiño R and Takashima F. 1995. Gonads. In: An Atlas of Fish Histology: Normal and Pathological Features (eds. Takashima F and Hibiya T). Kodansha, Tokyo, Japan. pp 144–150.

Raz E. 2000. The function and regulation of vasa-like genes in germ-cell development. Genome Biol. 1(3): reviews 1017.1

Takeuchi Y, Yoshizaki G, Kobayashi T, and Takeuchi T. 2002. Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the vasa gene promoter. Biol Reprod. 67: 1087–1092.

Takeuchi Y, Yoshizaki G, and Takeuchi T. 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod. 69: 1142–1149.

Takeuchi Y, Yoshizaki G, and Takeuchi T. 2004. Surrogate broodstock produces salmonids. Nature. 430: 629–630.

Thorgaard GH, Scheerer PD, and Zhang J. 1992. Integration of chromosome set manipulation and transgenic technologies for fishes. Mol Mar Biol Biotechnol. 1: 251–256.

Yano A, Suzuki K, and Yoshizaki G. 2008. Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout vasa regulatory regions. Biol Reprod. 78: 151–158.

Yoshida S, Nabeshima Y, and Nakagawa T. 2007. Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. Ann NY Acad Sci. 1120: 47–58.

Yoshizaki G, Sakatani S, Tominaga H, and Takeuchi T. 2000a. Cloning and characterization of a vasa-like gene in rainbow trout and its expression in the germ cell lineage. Mol Reprod Develop. 55: 364–371.

Yoshizaki G, Takeuchi Y, Kobayashi T, Ihara S, and Takeuchi T. 2002. Primordial germ cells: the blueprint for a piscine life. Fish Physiol Biochem. 26: 3–12.

Yoshizaki G, Takeuchi Y, Sakatani S, and Takeuchi T. 2000b. Germ-cell specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. Int J Dev Biol. 44: 323–326.

Part 6
Gene Transfer

Chapter 15

Spatial and Temporal Regulation of Transgene Expression in Fish

Ryan MacDonald and Marc Ekker

INTRODUCTION

The ability to express foreign DNA sequences into animals as transgenes has opened a wealth of possibilities in both basic and applied research. As all cells of an animal from an established transgenic line will contain the transgene, it is necessary to ensure that the transgene will only be expressed in those cells where its phenotypic effects are desired. Researchers rely on transcriptional control to spatially and temporally regulate transgenes. This is achieved through the identification and use of gene regulatory regions that confer tissue-specific expression to the transgene of interest. Here, we review methodological approaches that are used to transfer traits from one species to another in a tissue-specific manner. We will first examine some novel techniques for generating transgenic fish, discuss the necessary steps in establishing tissue-specific transgene expression, and finally, cover some recent efforts to develop systems for inducible transgene expression.

TRANSGENIC FISH

A transgene can be defined as a gene that is transferred from one organism to another. The DNA that is introduced in the recipient organism is often a genetically engineered construct and some or all its components will be from the foreign species. Gene constructs can be used to introduce desirable traits into an organism, and this ability to genetically modify a fish has many applications for aquaculture, environmental toxicology, pharmaceuticals, and developmental studies. The protein conferring the trait provided by the transgene can be encoded in genomic DNA fragments or in a complementary DNA. Most of the time, the transgene-encoded protein will be expressed in a species where it is not found endogenously. A transgenic approach can also be used for a protein that, although endogenous to the host species, is now expressed in cells in which it is not normally present or is expressed at higher levels than in nontransgenic animals. The transgene can sometimes be expressed concomitantly with a reporter gene, that is, a gene whose product can be easily detected. A classical example of a reporter gene is that coding for the green fluorescent protein. The reporter helps track the expression of the transgene of interest.

In fish, cytoplasmic injection of transgene DNA has been by far the most widely used approach to deliver transgenes. The first transgenic fish was generated in 1985 by Zhu et al. (1985) when a metallothionein promoter was used to drive expression of the human growth hormone gene in goldfish. Since

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

then, examples of transgene expression and transmission have been reported in many fish species: medaka, *Oryzias latipes* (Ozato et al. 1986); zebrafish, *Danio rerio* (Stuart et al. 1988; Ng et al., Chapter 18); Atlantic salmon, *Salmo salar* (Shears et al. 1991; Fletcher and Davies, Chapter 16); rainbow trout, *Oncorhynchus mykiss* (Chourrout et al. 1986); and Nile tilapia, *Oreochromis niloticus* (Wright and Pohaidak, Chapter 19).

PRIMARY TRANSGENIC ANIMALS AND ESTABLISHED TRANSGENIC LINES

The animal that results from injection of a gene construct is defined as a primary transgenic animal or founder. When a primary transgenic animal transmits the transgene to its progeny, a transgenic line is established. In primary transgenic fish, transgene expression has often been found to be mosaic, meaning that only a small portion of cells or tissues will contain and/or express the transgene. After microinjection of the construct, the one-cell embryo will continue to divide, each time dispersing the injected DNA between the daughter cells. Unless transgene integration into a host chromosome has occurred before the first cell division, DNA dispersal is not always uniform. Some cells may not contain any of the transgene DNA and a mosaic expression pattern will result. This also implies that the germ cells that give rise to the gametes will not necessarily have incorporated the transgene. As germline integration is an essential condition for transmission to future generations, mosaicism in primary transgenic fish results in low rates of transmission. In zebrafish, transmission of the transgene to the progeny ranges from 1–5% of injected fish (Nusslein-Volhard and Dahm 2002).

Despite these limitations, transient expression of a transgene allows for quick and easy assays of regulatory elements or to determine the phenotypic consequences of the misexpression of particular proteins (Rinder et al. 1992; Bajoghli et al. 2004).

TRANSPOSASE-MEDIATED TRANSGENESIS

When a transgene is transmitted through the germline, a copy of the transgene will be present in the genome of every cell of the progeny. This usually, but not always, alleviates mosaic expression. For years, researchers attempted to improve on the low rate of transgenesis from founder fish to progeny and found success with the use of transposons. Transposons are movable genetic elements that have been widely used for genetic studies in *Drosophila* and other organisms (Rubin and Spradling 1982; Kawakami 2007). The ability of transposons to move to new integration sites through the action of a transposase enzyme has made these genetic elements particularly useful for the production of transgenic animals. Such transposons also exist in vertebrate genomes but are often inactive. This has hindered their use in the development of transgenic vertebrates. Transposable elements have been identified in many species of fish (Izsvák et al. 1995). However, they do not appear to be suitable for genetic applications because of inactivation of the transposase genes by mutation (Izsvák et al. 1995; Ivics et al. 1996).

The Tol2 transposable element, which was first identified in the genome of medaka, appears to be the exception to the inactive transposable elements found in other vertebrate genomes. In fact, this element is autonomously active, meaning the element contains all of the proper sequences to transpose by itself (Kawakami et al. 1998). The Tol2 transposase gene encodes a protein that is fully functional and can transpose sequences flanked by Tol2 recognition sites (Kawakami and Shima 1999; Kawakami et al. 2000). The transposase protein not only facilitates excision of the sequence flanked by Tol2 sites, but will also insert this sequence into the genome (Fig. 15.1). This occurs via a cut and paste mechanism that will randomly integrate the sequence as a single copy. The effects of excision or insertion on the genome are minimal and limited to eight base pair duplications adjacent to the insertion site (Kawakami et al. 2000).

The use of Tol2-mediated transgenesis in fish has led to much improved rates of transmission to the progeny and reduced mosaicism in transgene expression. Fish injected with the transgene construct will transmit it to the progeny 30–70% of the time (Kawakami et al. 2004; Urasaki et al. 2006). Sequences of interest can be cloned between two Tol2 sequences. As there is no endogenous source of functional transposase in vertebrates, it must be supplied exogenously. The DNA construct containing

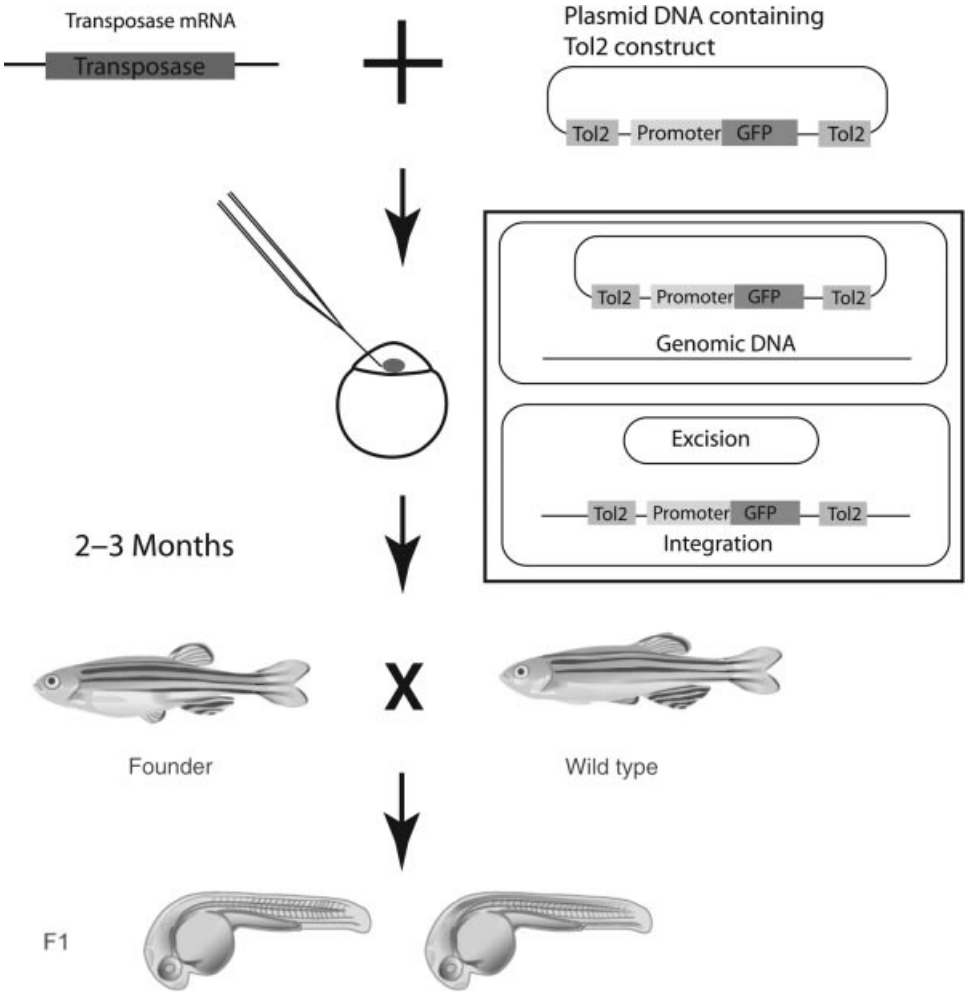


Figure 15.1. Generating transgenic zebrafish using Tol2-mediated transgenesis. The transposase mRNA is injected with the plasmid construct containing a spinal cord-specific promoter region and the reporter gene, green fluorescent protein (GFP), into fertilized eggs. The Tol2 construct will be excised from the plasmid before the promoter-GFP construct is inserted into the fish genome. The injected fish are grown to adulthood and mated for germline transmission of the transgene. The founder fish are mosaic and will have a mix of nontransgenic and hemizygous transgenic F1 progeny. (Figure modified from Kawakami 2007). (For color detail, please see the color plate section.)

the transgene is thus co-injected with the transposase mRNA that has been synthesized *in vitro*. Once injected, the Tol2-flanked transgene is excised by the transposase and integrated into the host's genome (Fig. 15.1). Integration is thought to occur early in embryonic development, resulting in the production of founder fish with reduced mosaicism. Since the transposase presence is transient, the transgene cannot be transposed to other sites in the genome later during the life of the fish or in its progeny.

The Tol2 system was developed in zebrafish and has become an integral part of the transgenesis research being carried out on this species. Successful transgene integration has also been obtained in medaka, frog, chicken, and mouse, as well as in human cells (see review by Kawakami 2007). Success in such diverse species may be due to the conservation among vertebrates of the factors necessary for transposition. Therefore, it is not unthinkable that this system will be transferable to fish species other than zebrafish. The increased efficiency of the Tol2-mediated transgenesis compared to the more traditional approaches has enabled large-scale transgenesis projects such as enhancer trapping, gene traps, and insertion mutagenesis screens (Kawakami et al. 2004; Parinov et al. 2004).

LIMITATIONS IN TRANSGENESIS EFFICIENCY

In addition to the above considerations on transgene transmission to the progeny, additional possible drawbacks must also be considered. One of the more frequently encountered complications is transgene concatemerization. It has frequently been observed that multiple transgene copies have been joined to each other to form a concatemer (Stuart et al. 1988; Yoshizaki et al. 1991). Concatemerization often results in low levels of transgene expression as observed in the rainbow trout (Iyengar and Maclean 1995). Concatemer expression has been shown to be silenced via heterochromatin formation (Dorer and Henikoff 1994). The same may apply in transgenic fish.

Integration of a transgene into the genome may also lead to its methylation, which will render the sequence transcriptionally inactive (Doerfler 1992). In mice, it has been noted that transgene concatemerization will lead to hypermethylation of the transgene (Mehtali et al. 1990). Studies have shown that a transgenic line may lose its ability to properly express the transgene after successive generations (Stuart et al. 1988, 1990). This loss of transgene expression through generations is likely attributable to methylation (Gibbs et al. 1994).

The location of the transgene insertion site in the genome can also affect its expression (Stuart et al. 1990; Lin et al. 1994). This can lead to variability between two independent lines of transgenic fish, both produced with identical gene constructs. This is known as the "position effect," where the genomic environment surrounding the transgene integration site will affect its expression. The presence, near the site of integration, of regulatory elements that regulate neighboring genes and also act on the transgene is often the cause of the position effect. Integration of a transgene into transcriptionally inactive chromatin such as heterochromatin constitutes another form of position effect. Finally, the insertion of the transgene can also disrupt an endogenous gene and lead to the development of a phenotype that is unrelated to the function of the transgene. This scenario was studied in mice where it was estimated to occur in 5–10% of integration events (Meisler 1992).

The number of transgene copies that will be integrated into the genome should also be considered. Both classical microinjection and Tol2-mediated transgenesis will yield multiple insertions, but via different processes. Classical microinjection will often result in multiple copies by concatemerization, as mentioned above. Transgenesis mediated by the Tol2 transposase does not cause this problem because the cut and paste mechanism facilitates single copy integration. However, the Tol2 vector is highly efficient and as a result can facilitate multiple insertions; for example, Urasaki et al. (2006) reported as many as 15 inserted copies in zebrafish.

The number of integrated transgene copies can potentially influence the overall levels of transgene expression and will likely have phenotypic consequences. Transgene integrant numbers can be determined by genomic Southern blot hybridization or by quantitative real-time PCR. If the production of a line of fish containing a single transgene copy is desired, it is recommended that a number of transgenic founder lines be established from which such a line can be selected.

Integration of a transgene into a predefined locus can eliminate variations between transgenic lines caused by the position effect. The targeted locus should be devoid of genomic influences such as heterochromatin and enhancer activity. Single copy insertions will remove variability caused by multiple insertions and concatemerization of the transgene. The Mos1-mediated single-copy insertion technique developed in *C. elegans* can accomplish these goals

(Frokjaer-Jensen et al. 2008). Similar systems for mouse and zebrafish are currently being developed.

REGULATION OF TRANSGENE EXPRESSION

It is often important to ensure expression of a transgene in specific cells at specific times. Therefore, the gene construct must contain the DNA sequence elements that will ensure the "desired" expression.

Eukaryotic gene expression is controlled at many levels. However, we will limit our discussion on transcriptional control to the *cis*-acting DNA regulatory elements. These regulatory elements can be manipulated to control the expression of a transgene at the transcriptional level. Transcription is initiated when an RNA polymerase recognizes and binds to the promoter sequence of a gene. We will call this sequence the "basal promoter" as some imprecision in terminology often results in confusion. The rate of transcription initiation of a basal promoter is influenced by a number of signals provided by additional regulatory sequences on the DNA. These regulatory sequences are the binding sites for transcription factors (TFs) that are responsible for the proper spatial and temporal expression of a gene. The overall levels of gene expression are conferred by the cumulative contributions of multiple TFs. Binding sites for TFs are often found in the first few hundred DNA base pairs located immediately upstream of the basal promoter. This region can be defined as the "promoter region" and is sometimes sufficient to confer proper gene regulation. For example, the common carp (*Cyprinus carpio*) *β-actin* promoter region has been shown to contain all the necessary regulatory elements for its expression (Liu et al. 1990). Similar studies have been carried out on the ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter (Butler and Fletcher 2009).

However, it has been well documented that expression of some genes also depends on the activity of additional DNA regulatory sequences, named enhancers. Enhancer elements are also the binding sites for TFs that stimulate gene expression. Enhancers have the ability to act over large genomic distances. They act independent of orientation and function independently of their position with respect to the basal promoter. Thus, enhancers can be located upstream, downstream, or even within the genes that they regulate (Fig. 15.2A).

Most enhancer sequences function in a temporally and spatially specific manner. This is achieved by enhancers only imparting their regulatory activity on a promoter if the correct combination of TFs are present. If the correct TFs are not present in the cellular environment, or if they are blocked from binding the DNA, the enhancer will remain inactive. One well-studied example in zebrafish is the *Sonic Hedgehog* gene (*shh*). This gene encodes a secreted peptide that controls a wide range of developmental processes. Mis-expression of this gene product has been implicated in the development of tumors and can act as a morphogen in different cells types (Ingham and McMahon 2001; McMahon et al. 2003). Thus, tight control of s*hh* gene expression is necessary for normal embryonic development. Several enhancers were identified within the *shh* locus, each controlling expression in specific tissues. These distinct enhancers combine to recapitulate most of the endogenous *shh* gene expression, indicating that each element is responsible for part of *shh* gene expression (Muller et al. 1999).

Additional regulatory DNA sequences such as silencers, repressor binding sites, and insulator sequences are also important for transcriptional control of gene expression. However, as they are not generally used to control transgene expression in a spatial or temporal manner, they will not be discussed further here.

IDENTIFICATION OF DNA REGULATORY ELEMENTS

Identification of the right complement of DNA regulatory sequences is the first step in the generation of a transgene that one wants to express in a tissue- and time-specific manner.

Comparisons of genomic DNA sequences from multiple species, also known as phylogenetic footprinting, have proven to be a very useful method to identify potential regulatory regions (Muller et al. 2002). The availability of genome sequences from evolutionarily distant species has made possible the identification of potential regions of functional significance. Because of the evolutionary forces, important functional regions of the genome will remain unaltered in distantly related genomes. Many

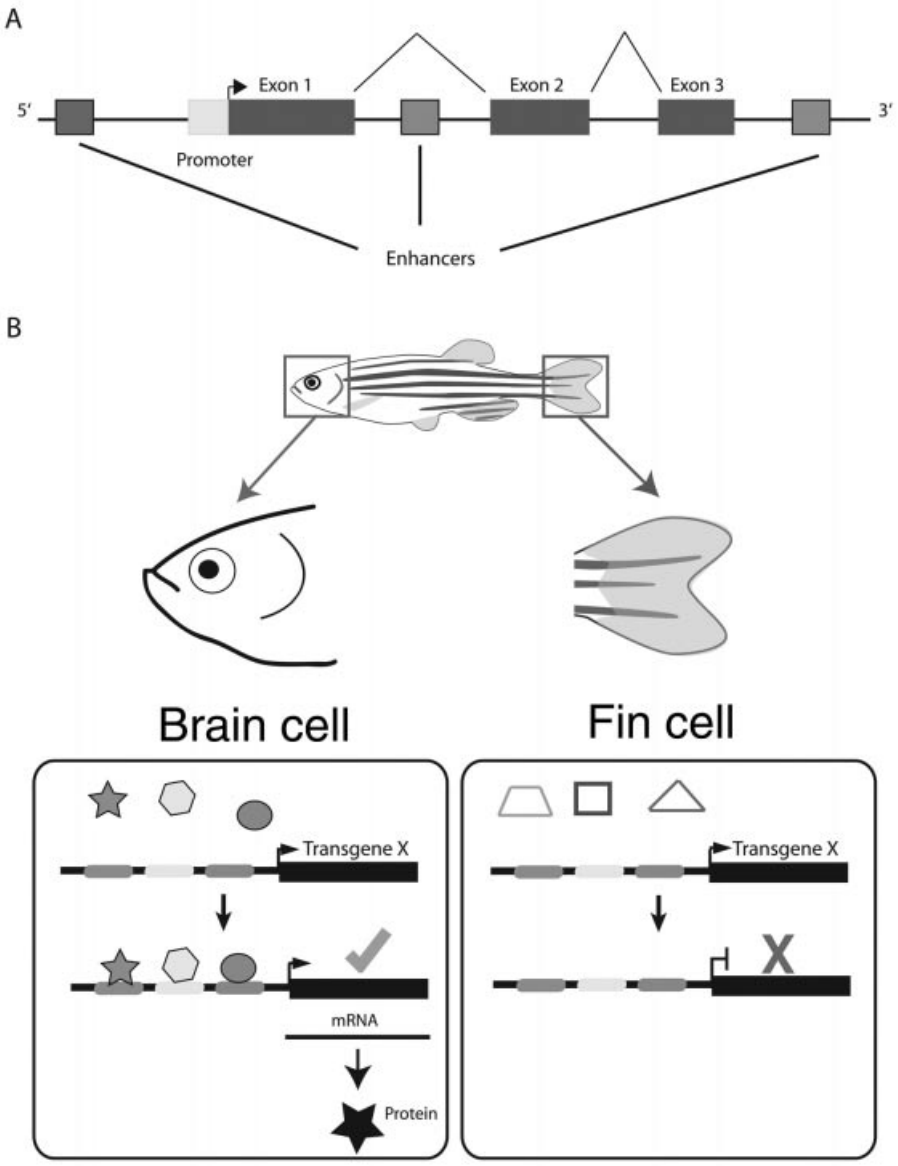


Figure 15.2. (**A**) Structure of a gene containing exons, a promoter region, and enhancers. The enhancers can be found upstream, within an intron, or downstream of the coding sequence. (**B**) Tissue-specific expression of a transgene can be obtained by generating a gene construct in which regulatory elements are controlling expression of the transgene of interest. Transcription factors will specifically bind DNA regulatory sequences based on their DNA-binding affinity preferences. In this example, the brain cells contain the proper combination of transcription factors to bind to regulatory sequences, whereas the fin cells do not. Transcription factor binding increases the frequency of transcription initiation at the promoter leading to higher mRNA and subsequent protein production within the cell. (For color detail, please see the color plate section.)

laboratories have identified regulatory regions with this approach and tested them in transgenic assays for regulatory function (Boeddrich et al. 1999; Zerucha et al. 2000). Once the regulatory function of a sequence is known, it can be used to control expression of various transgenes.

Regulatory elements important for proper gene expression are often located within a few kilobases of the gene of interest (Thomas 2003). However, these flanking regions are sometimes insufficient to mimic endogenous gene expression. This is due to the regulatory elements being located far from the basal promoter. Enhancer elements have been located up to one megabase from the transcription start site, thus complicating their identification (Lettice et al. 2003). One way to bypass the identification and characterization of regulatory elements is to use transgene constructs that encompass large genomic regions surrounding the gene of interest. This would maximize the probability of the transgene containing the necessary set of regulatory elements. Researchers have used vectors with the ability to house large genomic fragments of up to 300 kilobases. These vectors are called bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) (Shizuya et al. 1992; Ioannou et al. 1994). Cloning into these vectors using restriction enzymes is limited due to the large size of the inserts. However, a system of homologous recombination in bacteria has been developed that facilitates modifications of large DNA fragments (Copeland et al. 2001; Lee et al. 2001). Applications of this system include insertion of a reporter gene in frame with the endogenous gene, thus allowing the reporter to be expressed in the same tissue-specific manner as the gene of interest.

The use of BAC and PAC vectors to produce transgenic animals warrants additional consideration. The first use of BAC vectors to produce transgenic fish was reported in 1998 by Jessen et al. (1998). Their study reported a decrease in mosaicism and expression of the reporter gene that more closely mimicked endogenous expression than did a transgene produced by a recombinant plasmid that contained a shorter genomic DNA region. However, the production of transgenic fish with large constructs may also exacerbate the already low success rate of genomic integration (Yang et al. 2006). It is not unthinkable that transposon-mediated transgenesis would be suitable for BAC or PAC vectors and thus have the ability to transpose large DNA fragments.

HOMOLOGOUS AND HETEROLOGOUS REGULATORY SEQUENCES

Regulatory sequences from homologous (same species as the host) or heterologous origins can in theory be used to control the expression of a transgene. Promoter regions originating from viruses, bacteria, and mammals have all been used to produce transgenic fish. The necessity of using homologous regulatory sequences to drive transgene expression in fish has been the object of some discussion (Friedenreich and Schartl 1990; Bearzotti et al. 1992; Westerfield et al. 1992). It has been argued that the use of homologous DNA regulatory elements will be ideally suitable for the binding of host species TFs and thus produce optimal gene regulation. However, regulatory elements and their interactions with TFs have often been shown to be conserved over large evolutionary distances. It has also been suggested that the use of foreign sequences such as viral or bacterial DNA sequences may result in increased methylation and thus inactivation of the transgene (Challita and Kohn 1994; Chen et al. 2003).

Homologous promoter regions from a gene of interest have been shown to successfully mimic endogenous tissue-specific expression in transient and stable lines of transgenic fish (Rinder et al. 1992; Higashijima et al. 1997; Meng et al. 1997). Tissue-specific expression has also been seen with promoter regions and enhancers from the GATA-2 gene in transgenic zebrafish (Meng and Lin 2000). With the large amount of genomic sequence data available for many fish species, identifying a homologous sequence of interest can be determined readily. Thus, it is beneficial to search for a homologous promoter region to drive reporter expression before considering a heterologous source.

Heterologous Regulatory Sequences

A heterologous regulatory sequence is one which originates in the genome of one species and is used in another species. While heterologous promoters have been shown to be less desirable when generating transgenic fish, there are many opportunities for

them to be useful. Viral promoters are often classified as strong promoters and will provide ubiquitous expression of a transgene. Several viral promoters have been found to function in fish (Chong and Vielkind 1989; Powers et al. 1991). Mammalian promoter regions have had variable success in driving reporter expression in a proper spatial and temporal fashion in transgenic fish. Two mammalian *Hox* gene promoters had the ability to mimic endogenous expression in the nervous system and sclerotome of the zebrafish (Westerfield et al. 1992). Heterologous fish promoter regions are often useful for transgenesis. A promoter region from the winter flounder antifreeze protein gene expressed a transgene in a correct tissue-specific manner in transgenic Atlantic salmon (Hew et al. 1999). Similarly, an ocean pout antifreeze protein gene promoter drove the expression of a growth hormone transgene in rapidly growing transgenic Atlantic salmon (Hobbs and Fletcher 2008).

The *hsp70* promoter region has been tested in several heterologous scenarios. The *hsp70* from Mozambique tilapia (*Oreochromis mossambicus*) is functional in a common carp epithelioma papulosum cell line and in microinjected zebrafish (Molina et al. 2001). A comparative study between the *β-actin* promoter regions of carp and rat has shown that the carp promoter had higher expression levels than the equivalent rat promoter in rainbow trout and tilapia (Alam et al. 1996). In absence of homologous promoters, heterologous promoter regions can be useful to drive desired transgene expression.

Numerous developmental studies on transgenic fish have allowed researchers to identify various cell and tissue-specific regulatory elements. These regulatory elements drive gene expression in a wide range of tissues and have aided in the study of regulatory element function. For example, regulatory regions upstream of the *I-FABP* gene can target reporter gene expression to the zebrafish intestine (Her et al. 2004). The study of angiogenesis in vivo has been greatly facilitated by the *fli1* promoter region driving reporter expression in the embryonic vasculature (Lawson and Weinstein 2002). The development of cranial motor neurons could be followed during embryogenesis when reporter expression was targeted by an *islet1* regulatory region (Higashijima et al. 2000). These regulatory elements and many others can be further utilized to target transgene expression to a desired tissue (Fig. 15.2B).

Gal4-UAS

Tissue-specific expression of transgenes can also be accomplished using the dual Gal4-UAS system (Fig. 15.3) that has been extensively utilized in *Drosophila* and to a lesser extent in mice (Fischer et al. 1988; Ornitz et al. 1991). The yeast Gal4 regulatory protein will activate transcription of genes when bound to a specific recognition site called the upstream activating sequence (UAS). The Gal4 protein functions as a dimer and, once bound to the UAS, will activate transcription of the adjacent promoter (Carey et al. 1989). The Gal4 protein has been genetically modified to increase activity by pairing the Gal4 DNA-binding domain with the strong transcriptional activator (tTA) VP-16 (Sadowski et al. 1988). To produce tissue-specific expression of a transgene, production of the Gal4-VP16 protein can be targeted to specific cells by a set of regulatory elements (promoter region and/or enhancer). In a second transgene construct, the UAS sequence and a minimal promoter are fused with the gene of interest. Both of these constructs can be injected independently to generate distinct transgenic fish. By mating the two transgenic fish, Gal4-VP16 will induce expression of the transgene in a tissue-specific manner. This system can be especially useful when several different expression patterns of the transgene are necessary. The Gal4 construct can be paired with a new regulatory region, and a novel expression pattern can be generated. By generating a number of transgenic fish with different Gal4 expression patterns, the functions of different regulatory regions can be tested quite rapidly.

A particularly useful application of the Gal4 protein is in the generation of "transgenic trap lines." In zebrafish, the development of Tol2 transgenesis has greatly increased the ability of researchers to generate multiple stable transgenic lines. Zebrafish can now be utilized in enhancer and gene-trapping strategies similar to those used in *Drosophila* (Brand and Perrimon 1993; Scheer and Campos-Ortega 1999). In this experiment, the Gal4 protein-encoding gene is fused to a minimal promoter and microinjected into embryos. The Tol2-induced integration site of the transgene will be random within the genome. If

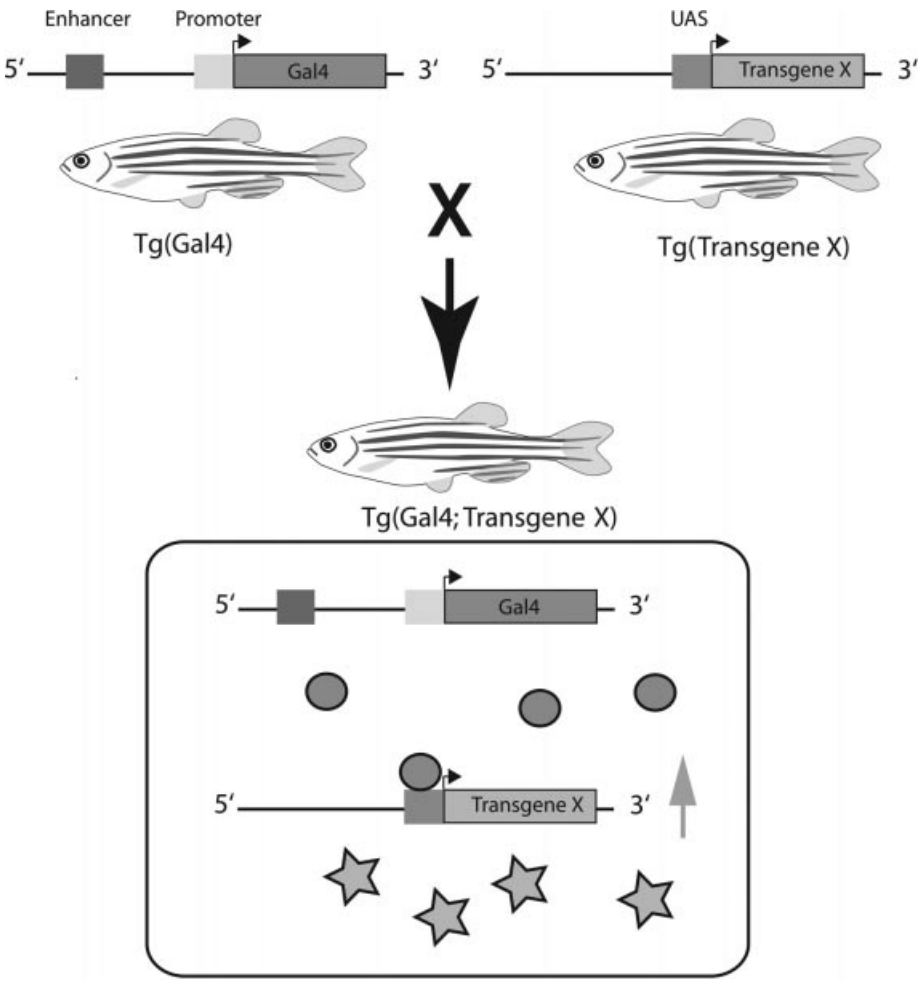


Figure 15.3. The Gal4-UAS binary system for tissue-specific expression of transgenes. The Gal4 transgene expression is controlled by tissue-specific regulatory elements (enhancer/promoter). The transgene of interest, Transgene X, is under the control of a Gal4-responsive regulatory element called upstream activating sequence (UAS). Individual transgenic fish are generated for each construct. These transgenic fish are mated to produce animals containing both constructs. The Gal4 protein (circles) will be produced in those cells where the regulatory elements are active and will bind to the UAS of Transgene X to stimulate its transcription. (For color detail, please see the color plate section.)

this transgene falls within the proximity of an active endogenous regulatory region, Gal4 will be transcribed in the corresponding temporal and spatial pattern. With the increased integration efficiency of the Tol2 vector, multiple Gal4 lines can be produced each with the potential to exhibit different expression patterns. Asakawa et al. (2008) generated Gal4 enhancer trap lines that were active in neural circuits of zebrafish. These neural circuits were then inhibited by a toxin-coding gene placed under the control of a UAS. This toxin was only produced in cells where Gal4 was expressed. This study illustrated

that large numbers of Gal4 enhancer trap lines can be generated efficiently and can be part of functional studies to address gene and cell function. Asakawa and colleagues (2008) have also modified the Gal4-VP16 TF to alleviate its toxicity in vertebrate cells (Gill and Ptashne 1988; Sadowski et al. 1988). The new Gal4FF molecule caused no obvious defects when injected into zebrafish embryos (Asakawa et al. 2008) and could greatly facilitate the use of this system in vertebrates, including other fish species.

INDUCIBLE GENE EXPRESSION SYSTEMS

Some applications of transgenesis necessitate the ability to induce expression of a transgene via an exogenous control mechanism. Inducible systems have also proven to be vital for many functional and developmental studies in other organisms. While systems for inducible gene expression have been available for several years, their utility in fish has been lagging due to technical difficulties. With the ongoing efforts to develop inducible systems in the zebrafish, there is hope that these systems will soon become available for use in other teleosts. A brief overview of three inducible systems, currently being tailored for piscine systems, is provided below.

Inducible systems used for controlled expression generally consist of two components: an activator, usually a TF whose expression is controlled in a tissue- or time-specific manner, and a second gene that contains a promoter region that is responsive to the activator. These components can be altered for use in transgenic systems. By generating independent transgenic fish lines with each construct, and by crossbreeding these transgenic fish lines, a Mendelian ratio of the progeny will contain both the activator and responsive promoter transgenes in the germline. These manipulations can be done with the tetracycline and Cre recombinase systems described below.

Tetracycline Regulated System

The system is designed using the regulatory elements of the tetracycline resistance operon of *Escherichia coli.* These elements bind to the tetracycline repressor (tetR) and negatively regulate transcription. When tetracycline is present, it binds to the tetR, resulting in the loss of its ability to bind to DNA and repress transcription. A transactivator protein called tetracycline-dependent tTA has been created by fusing the tetR DNA-binding domain with a strong transcriptional activation domain called VP16. This trans-activator has the ability to recognize the tetracycline regulatory elements (tetO; Fig. 15.4) in a tetracycline-dependent manner to activate transcription. The transgene of interest is then engineered to be controlled by the *tet* system. The gene of interest is placed under the control of the *tet* regulatory elements and a minimal promoter. Activation of the system requires binding of the tTA to the tetO. In the presence of tetracycline, the tTA is blocked from binding and there will be no transgene expression.

The tetracycline regulated system, when paired with a specific promoter to control the expression of tTA, can offer a tightly regulated, reversible, quantitative, temporal, and spatial control of transgene expression (Fig. 15.4). This system was first described by Gossen and Bujard (1992) by using mammalian HeLa cells. This system has since been utilized in *Drosophila*, mouse, and mammalian cell lines (Furth et al. 1994; Howe et al. 1995; Stebbins et al. 2001). The proper conditions for tight control over gene expression have been determined using the tetracycline system in fish cell culture (Muñoz et al. 2005).

Cyclization of Recombination (Cre)

Cre-mediated site-specific recombination has allowed for researchers to conditionally manipulate the mouse genome. This tool has become invaluable in its ability to alter genomic loci in vivo, and to conditionally control gene expression. Cre recombinase is a protein isolated from the bacteriophage P1, and it will recognize and catalyze DNA recombination between two recognition sequences (Hamilton and Abremski 1984; Hoess and Abremski 1984). These sequences, known as loxP sites, are 34 base pairs long. Any DNA sequence flanked by loxP sites (or "floxed") will be excised in the presence of Cre by recombination (Fig. 15.5). The use of Cre recombinase to produce tissue-specific and/or inducible transgene expression requires two independent transgenic lines: one with loxP sites flanking the sequence of interest and preventing its expression and another where the Cre recombinase protein is expressed under the control of tissue-specific regulatory elements.

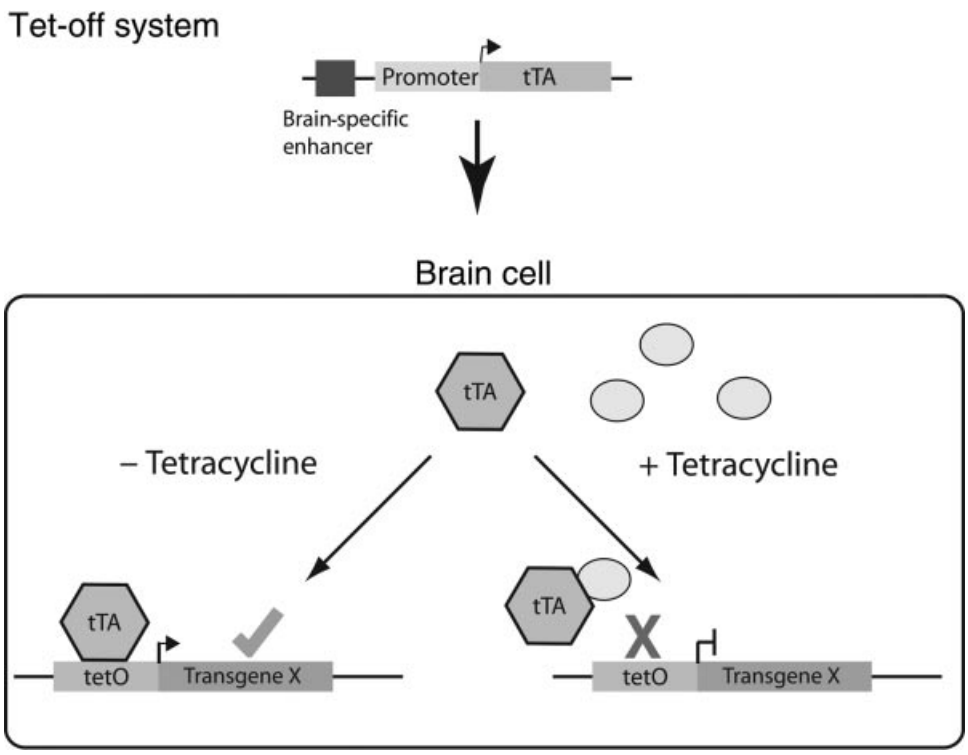


Figure 15.4. The tetracycline-off system for induction of transgene expression. Expression of the tetracycline activator, tTA, is driven by brain-specific regulatory elements. In the brain, the tTA is expressed and will bind to the tetracycline-induced promoter tetO, stimulating transcription of Transgene X. In the presence of tetracycline, the ability of the tTA to bind the tetO is blocked and Transgene X will not be transcribed. (For color detail, please see the color plate section.)

Cre can also be part of an inducible system in which it is part of a fusion protein, Cre-ERT, that contains a modified version of the ligand-binding domain (LBD) of the estrogen receptor (ER). The Cre-ERT is expressed either ubiquitously or in a tissue-specific manner but is unable to carry out the recombination event unless it is bound by tamoxifen, a synthetic drug that binds the modified ER LBD. The modification of the ER LBD makes it unable to bind endogenous estrogens. Therefore, only the administration of exogenous tamoxifen can induce Cre activity and thereby induce expression of the target gene placed between the loxP sites (Danielian et al. 1998). In mice, tamoxifen can be administered via intraperitoneal injection at the desired moment to induce the Cre activity. Intraperitoneal injection is probably the method of choice for large fish species.

The Cre recombinase system has been tested in zebrafish and has been shown to be a viable method for manipulating gene expression (Dong and Stuart 2004; Langenau et al. 2005; Pan et al. 2005). Thummel et al. (2005) generated transgenic zebrafish with Cre recombinase under the control of a heat shock promoter. When Cre expression was induced, it was able to excise a sequence from a transiently injected construct. These results indicate that Cre is functional in zebrafish cells.

Cytotoxicity attributable to Cre has been noted in a few mammalian systems (Schmidt et al. 2000; Loonstra et al. 2001). This cytotoxicity was not

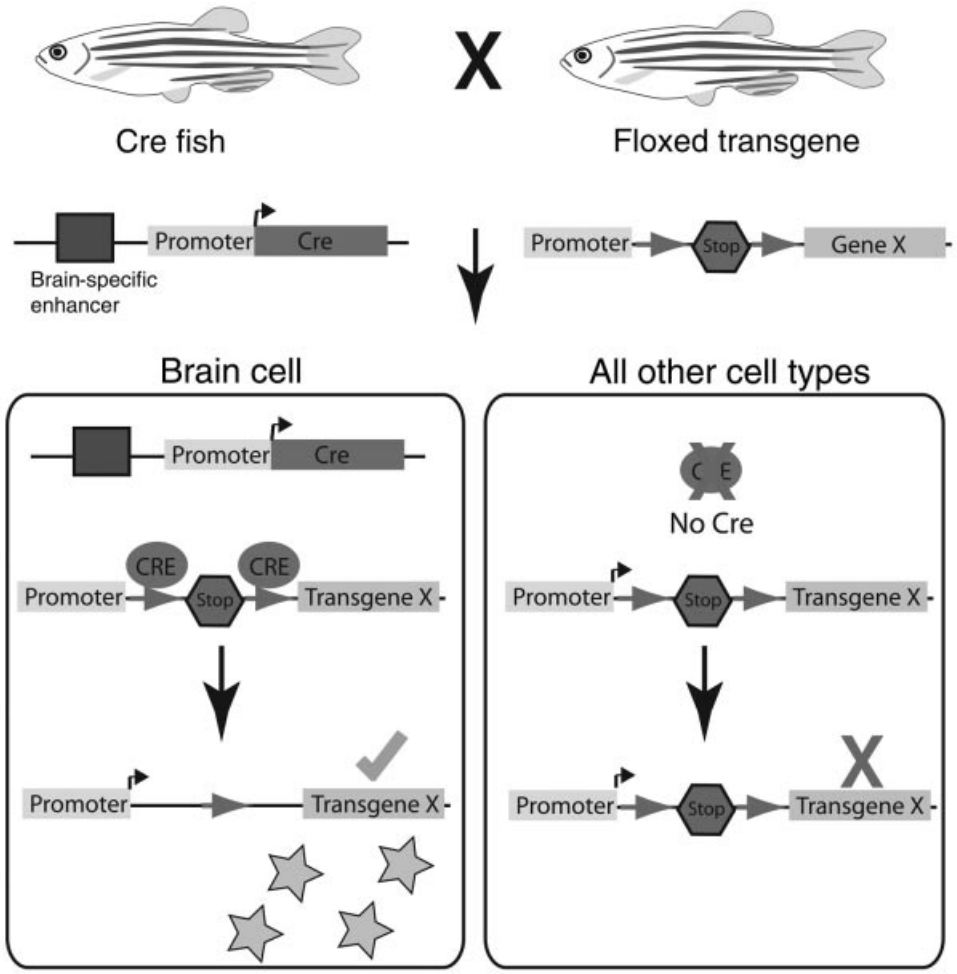


Figure 15.5. Activation of transgene expression by the Cre recombinase. Transgenic fish with cell-specific Cre expression are crossed to fish transgenic for a floxed Transgene X construct. Presence of a transcriptional stop or polyadenylation sequence (hexagon) between the floxed sequences prevents transcription of Transgene X. In transgenic fish containing both transgenes, Cre will recognize the loxP sequences (arrows) and excise the transcriptional stop sequence in a cell-specific manner. This allows for transcription to be initiated in Cre-expressing cells but not in others. (For color detail, please see the color plate section.)

experienced in zebrafish studies, as viable and fertile adults were identified after heat shock (Thummel et al. 2005). While this system has only been tested in zebrafish, its adaptation to other piscine models is only a matter of time.

Heat Shock

Inducible systems often work with an activator and response element, resulting in a system where two transgenic fish will need to be cross bred. Heat shock promoters are induced by endogenous factors,

resulting in the need for only one transgenic line. Poikilothermic animals such as insects, frogs, and fish are particularly suitable for heat shock-induced gene expression (D'Avino and Thummel 1999; Halloran et al. 2000; Wheeler et al. 2000). Mammalian systems are more difficult to use because their strict control over body temperature constitutes a limiting factor (Bajoghli et al. 2004).

Heat shock proteins (HSPs) are a family of proteins that are expressed when the cell undergoes stress (e.g., temperature increase). Their activation is a highly conserved process shared between prokaryotes and eukaryotes (Bardwell and Craig 1984; Liu et al. 1997). The heat shock promoter region contains a number of heat shock elements (HSE), which will be target sequences for HSPs (Bienz and Pelham 1987). In the absence of stress, the HSPs will remain as monomers with no DNA binding or transcriptional activation abilities. Once stressed, the HSPs will undergo a conformational change and trimerize, allowing them to activate transcription via the HSE (Wu 1995).

The heat shock response can be utilized as an inducible system and has been tested in medaka and zebrafish using heterologous heat shock promoter regions from frog and mouse (Adám et al. 2000; Grabher and Wittbrodt 2004). Homologous promoters from Mozambique tilapia, and artificial promoters, have also been tested (Molina et al. 2001; Bajoghli et al. 2004). The heat shock system is ideal when temporal control of a transgene is required. However, spatial control can be an issue because the whole fish is generally exposed to the stressor. There have been attempts to induce heat shock in localized areas in the zebrafish. Halloran et al. (2000) induced expression of a reporter gene driven by a heat shock promoter in single cells using a sublethal laser microbeam. The optical clarity and accessibility of zebrafish embryos make this method successful. Hardy et al. (2007) modified a soldering iron to induce transgene misexpression in a small area of the embryo. This method was found to be successful in inducing transgene expression in small groups of cells at various stages of development.

CONCLUSION

In the last few years, characterization of fish genomes has provided us with a rapidly growing selection of promoters/enhancers that can be used to target gene expression to specific cell types at certain times in the life of the animal or in response to certain physiological stimuli. Our understanding of the conservation of regulatory element activity across species is improving, and this will result in improved use of heterologous promoter sequences. Methodological developments such as transposon-mediated gene transfer are improving transgenesis in teleosts. Other methods developed in model species, including dual transgenes and transgene induction systems, enable faster and/or larger scale testing of transgenes. Although there are likely to be limitations to the adaptability of such approaches to transgenesis in commercially important fish species or in an aquaculture context, studies carried out in model systems with these methods will yield benefits by providing proofs of concept and valuable preliminary information on the biological consequences of transgene expression in teleosts.

REFERENCES

Adám A, Bártfai R, Lele Z, Krone PH, and Orbán L. 2000. Heat-inducible expression of a reporter gene detected by transient assay in zebrafish. Exp Cell Res. 256(1): 282–290.

Alam MS, Lavender FL, Iyengar A, Rahman MA, Ayad HH, Lathe R, Morlay SD, and MacLean N. 1996. Comparison of the activity of carp and rat B-actin gene regulatory sequence in tilapia and rainbow trout embryos. Mol Reprod Dev. 45: 117–122.

Asakawa K, Suster ML, Mizusawa K, Nagayoshi S, Kotani T, Urasaki A, Kishimoto Y, Hibi M, and Kawakami K. 2008. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proc Natl Acad Sci USA. 105(4): 1255–1260.

Bajoghli B, Aghaallaei N, Heimbucher T, and Czerny T. 2004. An artificial promoter construct for heat-inducible misexpression during fish embryogenesis. Dev Biol. 271(2): 416–430.

Bardwell JC and Craig EA. 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible dnaK gene are homologous. Proc Nat Acad Sci USA. 81(3): 848–852.

Bearzotti M, Perrot E, Michard-Vanhee C, Jolivet G, Attal J, Theron MC, Puissant C, Dreano M, Kopchick JJ, and Powell R. 1992. Gene expression following transfection of fish cells. J Biotechnol. 26(2–3): 315–325.

Bienz M and Pelham HR. 1987. Mechanisms of heat-shock gene activation in higher eukaryotes. Adv Genet. 24: 31–72.

Boeddrich A, Burgtorf C, Roest Crollius H, Hennig S, Bernot A, Clark M, and Reinhardt R. 1999. Analysis of the spermine synthase gene region in *Fugu rubripes, Tetraodon fluviatilis*, and *Danio rerio*. Genomics. 57: 164–168.

Brand AH and Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118(2): 401–415.

Butler TM and Fletcher GL. 2009. Promoter analysis of a growth hormone transgene in Atlantic salmon. Theriogenology. 72: 62–71.

Carey M, Kakidani H, Leatherwood J, Mostashari F, and Ptashne M. 1989. An amino-terminal fragment of GAL4 binds DNA as a dimer. J Mol Biol. 209(3): 423–432.

Challita PM and Kohn DB. 1994. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*. Proc Natl Acad Sci USA. 91: 2567–2571.

Chen ZY, Riu E, He CY, Xu H, and Kay MA. 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. Mol Ther. 8(3): 495–500.

Chong SSC and Vielkind JR. 1989. Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. Theor Appl Genet. 78(3): 369–380.

Chourrout D, Guyomard R, and Houdebine L. 1986. High efficiency gene transfer in rainbow trout (*Salmo gairdneririch*) by microinjection into egg cytoplasm. Aquaculture. 51: 143–150.

Copeland NG, Jenkins NA, and Court DL. 2001. Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet. 2(10): 769–779.

Danielian PS, Muccino D, Rowitch DH, Michael SK, and McMahon AP. 1998. Modification of gene activity in mouse embryos *in utero* by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 8(24): 1323–1326.

D'Avino PP and Thummel CS. 1999. Ectopic expression systems in *Drosophila*. Methods Enzymol. 306: 129–142.

Doerfler W. 1992. DNA methylation: eukaryotic defense against the transcription of foreign genes. Microb Pathog. 12: 1–8.

Dong J and Stuart GW. 2004. Transgene manipulation in zebrafish by using recombinases. Methods Cell Biol. 77: 363–379.

Dorer DR and Henikoff S. 1994. Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila*. Cell. 77: 993–1002.

Fischer JA, Giniger E, Maniatis T, and Ptashne M. 1988. GAL4 activates transcription in *Drosophila*. Nature. 332(6167): 853–856.

Friedenreich H and Schartl M. 1990. Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. Nucl Acids Res. 18: 3299–3305.

Frokjaer-Jensen C, Davis MW, Hopkins CE, Newman BJ, Thummel JM, Olesen SP, Grunnet M, and Jorgensen EM. 2008. Single-copy insertion of transgenes in *Caenorhabditis elegans*. Nature Genet. 40(11): 1375–1383.

Furth PA, St Onge L, Böger H, Gruss P, Gossen M, Kistner A, Bujard H, and Hennighausen L. 1994. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc Natl Acad Sci USA. 91: 9302–9306.

Gibbs PD, Peek A, and Thorgaard G. 1994. An *in vivo* screen for the luciferase transgene in zebrafish. Mol Mar Biol Biotechnol. 3(6): 307–316.

Gill G and Ptashne M. 1988. Negative effect of the transcriptional activator GAL4. Nature. 334: 721–724.

Gossen M and Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA. 89: 5547–5551.

Grabher C and Wittbrodt J. 2004. Efficient activation of gene expression using a heat-shock inducible Gal4/Vp16-UAS system in medaka. BMC Biotechnol. 4: 26–30.

Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, and Shoji W. 2000. Laser-induced gene expression in specific cells of transgenic zebrafish. Development. 127(9): 1953–1960.

Hamilton DL and Abremski K. 1984. Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. J Mol Biol. 178(2): 481–486.

Hardy ME, Ross LV, and Chien CB. 2007. Focal gene misexpression in zebrafish embryos induced by local heat shock using a modified soldering iron. Dev Dyn. 236(11): 3071–3076.

Her GM, Chiang CC, and Wu JL. 2004. Zebrafish intestinal fatty acid binding protein (I-FABP) gene promoter drives gut-specific expression in stable transgenic fish. Genesis. 38: 26–31.

Hew C, Poon R, Xiong F, Gauthier S, Shears M, King M, Davies P, and Fletcher G. 1999. Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. Transgenic Res. 8(6): 405–414.

Higashijima S, Hotta Y, and Okamoto H. 2000. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the *islet-1* promoter/enhancer. J. Neurosci. 20: 206–218.

Higashijima S, Okamoto H, Ueno N, Hotta Y, and Eguchi G. 1997. High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev Biol. 192(2): 289–299.

Hobbs RS and Fletcher GL. 2008. Tissue specific expression of antifreeze protein and growth hormone transgenes driven by the ocean pout (*Macrozoarces americanus*) antifreeze protein OP5a gene promoter in Atlantic salmon (*Salmo salar*). Transgenic Res 17: 33–45.

Hoess RH and Abremski K. 1984. Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. Proc Natl Acad Sci USA. 81(4): 1026–1029.

Howe JR, Skryabin BV, Belcher SM, Zerillo CA, and Schmauss C. 1995. The responsiveness of a tetracycline-sensitive expression system differs in different cell lines. J Biol Chem. 270: 14168–14174.

Ingham PW and McMahon AP. 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15: 3059–3087.

Ioannou PA, Amemiya CT, Garnes J, Kroisel PM, Shizuya H, Chen C, Batzer MA, and de Jong PJ. 1994. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nat Genet. 6(1): 84–89.

Ivics Z, Izsvak Z, Minter A, and Hackett PB. 1996. Identification of functional domains and evolution of Tc1-like transposable elements. Proc Natl Acad Sci USA. 93(10): 5008–5013.

Iyengar A and Maclean N. 1995. Transgene concatemerisation and expression in rainbow trout (*Oncorhynchus mykiss*). Mol Mar Biol Biotechnol. 4: 248–254.

Izsvák Z, Ivics Z, and Hackett PB. 1995. Characterization of a Tc1-like transposable element in zebrafish (*Danio rerio*). Mol Gen Genet. 247(3): 312–322.

Jessen JR, Meng A, McFarlane RJ, Paw BH, Zon LI, Smith GR, and Lin S. 1998. Modification of bacterial artificial chromosomes through chi-stimulated homologous recombination and its application in zebrafish transgenesis. Proc Natl Acad Sci USA. 95(9): 5121–5126.

Kawakami K. 2007. Tol2: a versatile gene transfer vector in vertebrates. Genome Biol. 8(Suppl 1): S7.

Kawakami K and Shima A. 1999. Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. Gene. 240(1): 239–244.

Kawakami K, Koga A, Hori H, and Shima A. 1998. Excision of the tol2 transposable element of the medaka fish, *Oryzias latipes,* in zebrafish, *Danio rerio*. Gene. 225(1–2): 17–22.

Kawakami K, Shima A, and Kawakami N. 2000. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proc Natl Acad Sci USA. 97(21): 11403–11408.

Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, and Mishina M. 2004. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Dev Cell. 7(1): 133–144.

Langenau DM, Feng H, Berghmans S, Kanki JP, Kutok JL, and Look AT. 2005. Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA. 102(17): 6068–6073.

Lawson ND and Weinstein BM. 2002. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 248: 307–318.

Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, and Copeland NG. 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics. 73(1): 56–65.

Lettice LA, Heaney SJ, Purdie LA, Li L, de Beer P, Oostra BA, Goode D, Elgar G, Hill RE, and de Graaff E. 2003. A long-range *Shh* enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. Hum Mol Genet. 12(14): 1725–1735.

Lin S, Gaiano N, Culp P, Bums JC, Friedman T, Yee JK, and Hopkins N. 1994. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science. 265: 666–669.

Liu, XD, Liu PC, Santoro N, and Thiele DJ. 1997. Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF. EMBO J. 16(21): 6466–6477.

Liu ZJ, Moav B, Faras AJ, Guise KS, Kapuscinski AR, and Hackett PB. 1990. Functional analysis of elements affecting expression of the beta-actin gene of carp. Mol Cell Biol. 10(7): 3432–3440.

Loonstra A, Vooijs M, Beverloo HB, Allak BA, van Drunen E, Kanaar R, Berns A, and Jonkers J. 2001. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc Natl Acad Sci USA. 98(16): 9209–9214.

McMahon AP, Ingham PW, and Tabin CJ. 2003. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol. 53: 1–114.

Mehtali M, LeMeur M, and Lathe R. 1990. The methylation free status of a housekeeping transgene is lost at high copy number. Gene. 91: 179–184.

Meisler MH. 1992. Insertional mutation of "classical" and novel genes in transgenic mice. Trends Genet. 8(10): 341–344.

Meng A, Tang H, Ong BA, Farrell MJ, and Lin S. 1997. Promoter analysis in living zebrafish embryos identifies a *cis*-acting motif required for neuronal expression of GATA-2. Proc Natl Acad Sci USA. 94(12): 6267–6272.

Meng A and Lin S. 2000. Tissue-specific expression of GFP reporter gene in germline driven by GATA-2 promoter and enhancers in zebrafish. Chinese Sci Bull. 45(1): 31–34.

Molina A, Di Martino E, Martial JA, and Muller M. 2001. Heat shock stimulation of a tilapia heat shock protein 70 promoter is mediated by a distal element. Biochem J. 356(2): 353–359.

Muller F, Blader P, and Strahle U. 2002. Search for enhancers: teleost models in comparative genomic and transgenic analysis of *cis*-regulatory elements. BioEssays. 24: 564–572.

Muller F, Chang B, Albert S, Fischer N, Tora L, Strähle U. 1999. Intronic enhancers control expression of zebrafish sonic hedgehog in floor plate and notochord. Development. 126(10): 2103–2116.

Muñoz I, Carrillo M, Zanuy S, and Gómez A. 2005. Regulation of exogenous gene expression in fish cells: an evaluation of different versions of the tetracycline-regulated system. Gene. 363: 173–182.

Nusslein-Volhard K and Dahm R (eds). 2002. Zebrafish: a practical approach. Oxford University Press, Oxford.

Ornitz DM, Moreadith RW, and Leder P. 1991. Binary system for regulating transgene expression in mice: targeting *int-2* gene expression with yeast GAL4/UAS control elements. Proc Natl Acad Sci USA. 88: 698–702.

Ozato K, Kondoh H, Inohara H, Iwamatsu T, Wakamatsu Y, and Okada TS. 1986. Production of transgenic fish: introduction and expression of chicken delta-crystallin gene in medaka embryos. Cell Differ. 19(4): 237–244.

Pan X, Wan H, Chia W, Tong Y, and Gong Z. 2005. Demonstration of site-directed recombination in transgenic zebrafish using the Cre/loxP system. Transgenic Res. 14(2): 217–223.

Parinov S, Kondrichin I, Korzh V, and Emelyanov A. 2004. Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes *in vivo*. Dev Dyn. 231(2): 449–459.

Powers DA, Gonzalez-Villasenor LI, Zhang PJ, Chen TT, and Dunham RA. 1991. Studies on transgenic fish: gene transfer, expression, and inheritance. Bio/Technology. 16: 307–324.

Rinder H, Bayer TA, Gertzen EM, and Hoffmann W. 1992. Molecular analysis of the ependymin gene and functional test of its promoter region by transient expression in *Brachydanio rerio*. DNA Cell Biol. 11(6): 425–432.

Rubin GM and Spradling AC. 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science. 218(4570): 348–353.

Sadowski I, Ma J, Triezenberg S, and Ptashne M. 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature. 335(6190): 563–564.

Shears MA, Fletcher GL, and Hew CL. 1991. Transfer, expression and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). Mol Mar Biol Biotechnol. 1(1): 58–63.

Scheer N and Campos-Ortega JA. 1999. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mech Dev. 80(2): 153–158.

Schmidt EE, Taylor DS, Prigge JR, Barnett S, and Capecchi MR. 2000. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc Natl Acad Sci USA. 97(25): 13702–13707.

Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, and Simon M. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc Natl Acad Sci USA. (18): 8794–8797.

Stebbins MJ, Urlinger S, Byrne G, Bello B, Hillen W, and Yin JC. 2001. Tetracycline-inducible systems for *Drosophila*. Proc Natl Acad Sci USA. 98: 10775–10780.

Stuart GW, McMurray JV, and Westerfield M. 1988. Replication, integration and stable germ-line

transmission of foreign sequences injected into early zebrafish embryos. Development. 103(2): 403–412.

Stuart GW, Vielkind JR, McMurray JA, and Westerfield M. 1990. Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. Development. 109(3): 577–584.

Thomas C. 2003. Cell biology: enlightened messages. Nature. 426(6962): 27.

Thummel R, Burket CT, Brewer JL, Sarras MP, Li L, Perry M, McDermott JP, Sauer B, Hyde DR, and Godwin AR. 2005. Cre-mediated site-specific recombination in zebrafish embryos. Dev Dyn. 233(4): 1366–1377.

Urasaki A, Morvan G, and Kawakami K. 2006. Functional dissection of the Tol2 transposable element identified the minimal *cis*-sequence and a highly repetitive sequence in the subterminal region essential for transposition. Genetics. 174(2): 639–649.

Westerfield M, Wegner J, Jegalian BG, DeRobertis EM, and Püschel AW. 1992. Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. Genes Dev. 6(4): 591–598.

Wheeler GN, Hamilton FS, and Hoppler S. 2000. Inducible gene expression in transgenic *Xenopus* embryos. Curr Biol. 10(14): 849–852.

Wu C. 1995. Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol. 11: 441–469.

Yang Z, Jiang H, Chachainasakul T, Gong S, Yang XW, Heintz N, and Lin S. 2006. Modified bacterial artificial chromosomes for zebrafish transgenesis. Methods. 39(3): 183–188.

Yoshizaki G, Oshiro T, and Takashima E. 1991. Introduction of carp alpha-globin gene into rainbow trout. Nippon Suisan Gakkaishi. 57: 819–824.

Zerucha T, Stuhmer T, Hatch G, Park BK, Long Q, Yu G, Gambarotta A, and Ekker M. 2000. A highly conserved enhancer in the *Dlx5/Dlx6* intergenic region is the site of cross-regulatory interactions between *Dlx* genes in the embryonic forebrain. J Neurosci. 20: 709–721.

Zhu Z, Li G, He L, and Chen S. 1985. Novel gene transfer into the fertilized eggs of gold fish (*Carassius auratus* L. 1758). J Appl Ichthyol. 1(1): 31–34.

Chapter 16

Antifreeze Protein Gene Transfer—Promises, Challenges, and Lessons from Nature

Garth L. Fletcher and Peter L. Davies

INTRODUCTION

One of the major impediments to expanding fish aquaculture into cold and, at times, icy coastal marine regions of the world is the danger of mortalities due to freezing. Nature's solution to this problem lies with fish that produce antifreeze proteins, thus enabling them to reside in such environments. The question is: can nature's solution to avoiding freezing be duplicated by antifreeze protein gene transfer? We started out to do this in the 1980s using the knowledge and genes that we had on hand at that time. Although we were successful in transferring AFP genes to the Atlantic salmon, the expression levels were too low to improve their freeze resistance. Over the ensuing 20 years of research, we have accumulated a great deal more knowledge about AFP gene expression and the mechanisms whereby AFP prevent ice formation within tissues of ice-laden water residents. What is clear from this research is that producing freeze-resistant fish is a much more complex problem than we thought in the 1980s. This chapter will summarize our progress toward producing more freeze-resistant salmon and point to hurdles that need to be overcome before complete success can be achieved.

THE DANGER POSED TO FISH BY LOW SEA WATER TEMPERATURES

The colligative freezing point of teleost fishes' body fluids approximates -0.6 to -0.8°C, while the freezing point of sea water can be as low as -2.0°C, depending on the salinity. Since most teleosts are ectotherms, their body temperatures are identical to that of their environment. Therefore, the temperature of fish residing in polar or subpolar oceans can be a degree or more below the freezing point of their body fluids. When fish are in this unstable state, ice crystal contact can immediately nucleate freezing in the fish and result in death. Most fish avoid such conditions; however, some have adapted to these environments by evolving freeze resistance mechanisms, the most studied of which are the antifreeze proteins (Fletcher et al. 2001).

ANTIFREEZE PROTEINS

The early work of deVries et al. (1970) established the presence of antifreeze glycoproteins in the serum of Antarctic fish. Since that time, four additional antifreeze protein types differing in carbohydrate content, amino acid composition, protein sequence, and tertiary structure have been isolated from tissues

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

of a diverse group of marine teleosts (Table 16.1). These antifreeze proteins and glycoproteins are collectively abbreviated as AF(G)P.

All of the AF(G)P have an affinity for ice and exert their effect on the freezing point of aqueous solutions by a mechanism known as adsorption–inhibition (Raymond and DeVries 1977). The antifreeze proteins act by binding to specific planes of ice and inhibiting the addition of water molecules to those planes. By stopping ice growth on those planes, the AF(G)P modify the ice crystal habit such that it typically grows into a hexagonal bipyramid (Fig. 16.1). This inhibition of ice crystal growth and the resulting freezing point depression are thought to be the major means by which the AF(G)P protect the body fluids of fish from freezing.

Table 16.1. Antifreeze Proteins Isolated from Fish.

AF(G)P	Repeats	2° Structure	Major Isoforms	3° Structure	Progenitor Type
AFGP	Yes	Extended coil	Range of sizes (2.6–34 kDa)	Polyproline type II helix?	Repeat sequence within trypsinogen gene
Type I	Yes	Amphipathic α -helix	3–9 kDa with liver and skin specific isoforms	Single α-helix	Unknown*
			34 kDa hyperactive	Homodimer of α-helices	
Type II	No	Mixed α and β	Ca^{2+}-dependent	globular	C-type lectin
			Ca^{2+}-independent (14 kDa)		
Type III	No	Mixed α and β	SP isoforms	β-clip fold	Sialic acid synthase
			QAE isoforms (6–7 kDA)		
Type IV	No	α-helix	None (12 kDA)	4-helix bundle	Lipoprotein

AFGPs are made up of a tripeptide repeat of Ala-Ala-Thr with a disaccharide O-linked to the Thr. The different isoform lengths result from proteolyic processing of polyprotein precursors (Chen et al. 1997). AFGP are present in Antarctic notothenids and northern gadidae (Davies et al. 1999). Type I AFPs are alanine-rich (>60%) and present in righteye flounders, sculpin, and lumpsuckers (Davies et al. 1989; Evans and Fletcher 2001).

*Evans and Fletcher (2005b) suggest that the progenitor for type I AFP found in lumpsuckers could be egg chorion proteins or type II keratin. The two different isoforms of type II AFP (Ca^{2+}-dependent and Ca^{2+}-independent) are found in three different taxanomic groups of fish: smelt, herring, and sea raven (Ewart and Fletcher 1990). These AFP may have been dispersed by horizontal gene transfer (Graham et al. 2008b). Type III AFPs, which are widely distributed within the suborder Zoarcoidei (Shears et al. 1993) have been best characterized in wolffish and ocean pout (Hew et al. 1988). The protein sequence homology between type III AFP and the C-terminal region of mammalian sialic acid synthase is suggestive of its evolutionary origins (Baardness and Davies 2001). The SP- and QAE-isoforms of type III AFP have very different *pI* values and can be found together in the same fish (Hew et al. 1988). The QAE-isoforms confer thermal hysteresis activity to the SP-isoforms when mixed together (Nishimiya et al. 2005). The type IV AFP present in longhorn sculpin does not appear to serve as an antifreeze protein but it fortuitously has the ability to bind to ice (Deng et al. 1997; Gauthier et al. 2008).

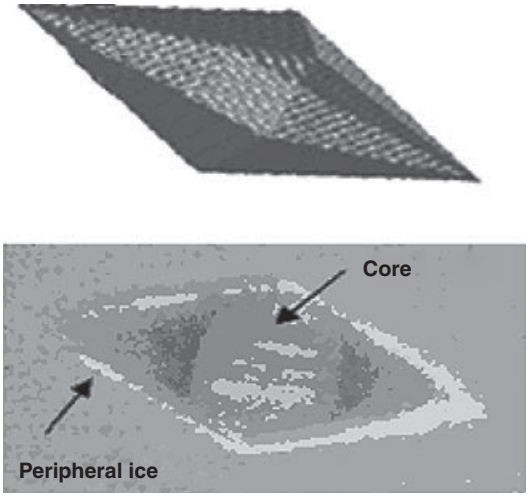


Figure 16.1. Illustration of AFP binding to ice. Ice crystals were grown in the presence of type III AFP conjugated to green fluorescent protein (GFP). The upper panel shows a model of the bipyramidal ice crystal shape. The lower panel illustrates AFP binding (light colored region) to the surface of the ice as evidenced using fluorescence microscopy (Pertaya et al. 2007). Note that the core of the ice crystal is highly fluorescent due to the incorporation of AFP-GFP during the initial flash-freezing. The ice was melted back to a single, roughly spherical crystal before being slowly grown into the bipyramidal ice crystal shape where AFP-GFP is concentrated on the binding planes.

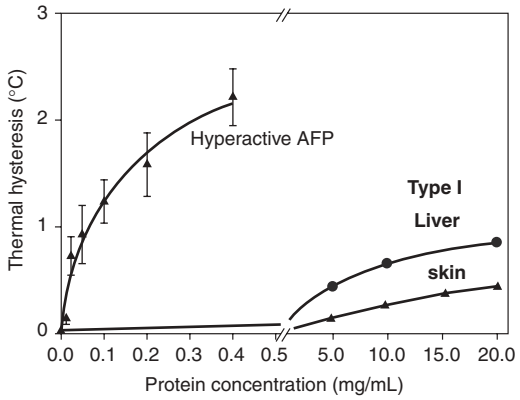


Figure 16.2. Comparison of type 1 skin, liver, and hyperactive AFP antifreeze protein activities (thermal hysteresis) as a function of AFP concentration. (Data taken from Gong et al. 1996; Marshall et al. 2004b)

Since the AF(G)P act by binding to ice, their freeze depressing action is noncolligative, while their effect on the melting point remains largely colligative. Therefore, there can be a considerable difference between the freezing and melting temperatures of aqueous solutions that contain antifreeze proteins. This temperature difference is referred to as thermal hysteresis and values obtained are a function of AF(G)P concentration (Fig. 16.2).

The relative importance of AF(G)P and the colligative properties (due largely to NaCl) of blood plasma to the freeze resistance of fish currently being cultured or being considered for culture in marine waters of Atlantic Canada is illustrated in Fig. 16.3. The freezing point of species that do not possess AF(G)P, such as Atlantic salmon, rainbow trout, Arctic charr, and halibut, is solely dependent on the colligative properties of their body fluids (-0.72°C to -0.79°C), whereas species such as winter flounder, ocean pout, and Atlantic wolffish with high levels of AF(G)P are protected from freezing down to temperatures at, or lower than, the freezing point of sea water. Other species with lower levels of AF(G)P have a more limited improvement in their resistance to freezing (yellowtail flounder, Atlantic cod).

WINTER FLOUNDER: A MODEL FOR ANTIFREEZE PROTEIN FREEZE RESISTANCE STRATEGIES

We have chosen the winter flounder as a model example of how marine teleosts avoid freezing because it is the species about which we know most. The winter flounder is a resident of inshore coastal waters of Atlantic Canada and northeastern United States (Scott and Scott 1988). In Newfoundland, it is subjected to seasonally cycling temperatures ranging from 14–16°C during summer to -1.8°C during February and March. During the winter months, the areas inhabited by the winter flounder are

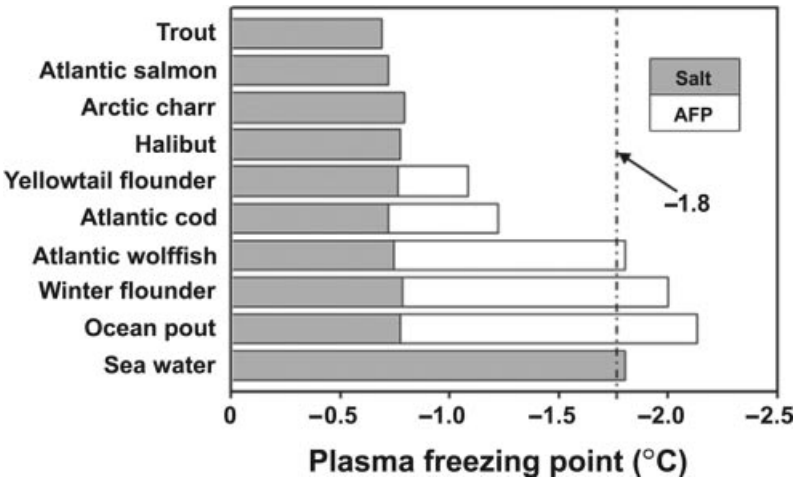


Figure 16.3. The contribution of antifreeze proteins to the freeze protection of fish. The plasma freezing points of nine teleost fish from Atlantic Canada are compared with the freezing point of sea water (−1.8°C). Plasma freezing points closely approximate the whole fish freezing point. The shaded portion of each bar represents the colligative freezing point, while the open portion represents the enhancement of freeze protection attributable to antifreeze proteins. Fish species: trout, *Oncorhynchus mykiss*; Atlantic salmon, *Salmo salar*; Arctic charr, *Salvelinus alpinus*; halibut, *Hippoglossus hippoglossus*; yellowtail flounder, *Limanda ferruginea*; Atlantic cod, *Gadus morhua*; Atlantic wolffish, *Anarhichas lupus*; winter flounder, *Pseudopleuronectes americanus*; and ocean pout, *Macrozoarces americanus.*

frequently covered in ice where storm activity can drive ice crystals deep into the water column. Thus, the winter flounder inhabits an environment that would be lethal to most fish. However, it survives these freezing conditions by synthesizing three distinct isoforms of alanine-rich type 1 AFP encoded by three homologous gene families: the classic liver type that are produced by the liver and secreted into the circulatory system (Pickett et al. 1984), the skin type that are expressed predominantly in the external epithelia (Gong et al. 1996), and the more recently discovered hyperactive AFP that is also produced by the liver (Marshall et al. 2005; Table 16.1). The relative potency of the three AFP species is illustrated in Figure 16.2. Several other species such as ocean pout, wolfish, and lumpsucker (*Liparis* sp) are also known to synthesize AFP in both liver and external epithelia (Gong et al. 1992; Evans and Fletcher 2004, 2005a; Desjardins et al. 2006, unpublished data).

The winter flounder limits the production and secretion of the liver-type AFP into the plasma in the winter months, a strategy that likely reduces the metabolic costs associated with AFP synthesis. This annual cycle of AFP production correlates closely with the annual cycle of sea water temperature. There is little or no AFP present in the plasma during the summer. However, during autumn, as the water temperatures decline and the day lengths shorten, AFP gene transcription is initiated by the liver, followed by secretion of the 3–4 kDa AFP into the plasma to reach concentrations of 10–15 mg/mL during the coldest winter months. With the approach of spring, liver AFP mRNA levels decline and the AFP are rapidly cleared from the plasma as the temperature increases above 0°C (Fig. 16.4). Peak winter levels of both the small and larger AFP increase the freeze resistance of winter flounder to temperatures below that of sea water (−1.8°C; Fig. 16.3). The small 3–4 kDa AFP secreted into the circulatory

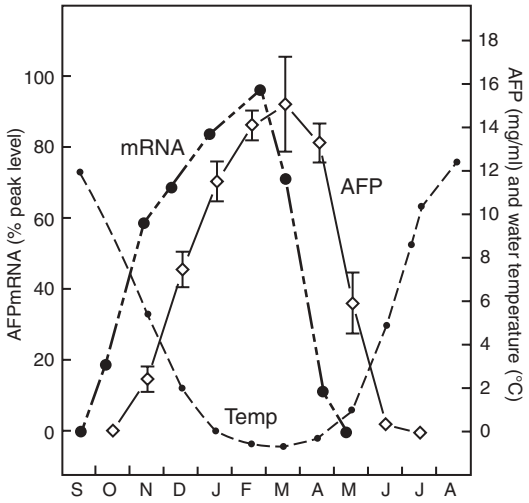


Figure 16.4. Seasonal changes in antifreeze protein (AFP) and AFP mRNA levels in Newfoundland winter flounder. Plasma AFP levels (open circles) are plotted as means of 5–10 plasma samples ± SE. AFP mRNA levels (black circles) are plotted as a percentage of the maximum level measured. Seawater temperatures are indicated by the dotted line. (Figure drawn from data presented in Fletcher et al. 1992 and Davies et al. 1999.)

system by the liver would be distributed rapidly throughout much of the extracellular space, including the urine and bile where they would serve to bind to nascent ice crystals that may form within or propagate into the fish (Fletcher et al. 1989). The larger 34 kDa AFP that are also synthesized by the liver and exported to the plasma during winter (Young and Fletcher 2008) are manyfold more active than the smaller ones at reducing the freezing temperature of the plasma (Fig. 16.2). However, these AFP function as relatively large, highly asymmetrical 32 kDa dimers that are 275 Å in length (Graham et al. 2008a), thus their distribution within the extracellular space may be more limited than the smaller AFP.

A second freeze resistance strategy exhibited by the winter flounder is the production of AFP by the skin-type AFP gene family in the external epithelia of gills, skin, and digestive tract. These AFP could serve to protect such tissues from the damaging effects of freezing should they come into direct contact with external ice. Skin-type AFP genes differ from the smaller liver-type AFP in that they lack pre- and proAFP sequences (Gong et al. 1996). This lack of the presignal sequence suggests that these AFP may remain and function inside the cell. However, histological studies have demonstrated that the functional location of the AFP can differ between tissues (Murray et al. 2002, 2003). For example, an in situ hybridization investigation demonstrated the expression of AFP mRNA in cells distributed throughout the skin epithelia, gill filaments, and lamella. However, an immunochemical examination of the skin epithelial cells revealed that AFP was localized in the interstitial space in close association with the cell membrane and absent from the cytoplasm. In contrast, AFP was only observed in the cytoplasm of the gill epithelial cells (Fig. 16.5).

Since the fish skin epithelial cell layer is avascular, it is possible that the small liver-type AFP cannot readily diffuse into this tissue from the circulatory system (Bullock and Roberts 1974; Burton and Fletcher 1983). Therefore, the production of AFP by the epithelial cells and its release into the interstitial space could be a major reason why the winter flounder skin is such an effective barrier to ice propagation (Valerio et al. 1992). The interstitial AFP could also protect the epithelial cells directly by preventing nascent ice crystal formation and growth within the interstitial space, a process that would promote increased osmolality and consequently cell dehydration due to the efflux of water.

The functional significance of the intracellular AFP in the gill lamellae epithelia is more difficult to evaluate. Two hypotheses come to mind. The first is that there is a need to noncolligatively lower the freezing point of the gill epithelial cells and thus prevent the formation of intracellular ice by external nucleation, or through gap junctions connecting contiguous cells that could be damaged (Bartels and Potter 1993; Larese et al. 1996; Sandbacka et al. 1998; Murray et al. 2002).

The second hypothesis stems from the fact that gill epithelial cells have a rapid turnover rate. Thus during apoptosis, the AFP could be released into the layer of mucous covering the epithelia where, in

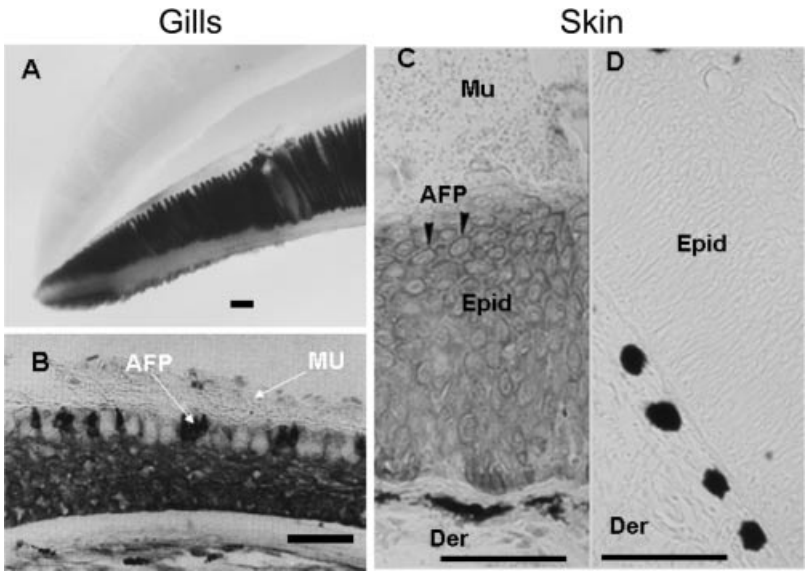


Figure 16.5. Tissue and cellular localization of AFP production in gill and skin epithelia of Newfoundland winter flounder. (**A**) Whole-mount in situ hybridization showing distribution of skin type AFP mRNA expression (dark stain) on gill filament lamellae. The lightly stained lamellae served as the control (Murray et al. 2002). (**B**) High magnification of a gill filament showing the cellular distribution of AFP-producing cells. (**C**) Extracellular localization of skin type AFP in the epidermis of winter flounder. (**D**) Control skin. All scale bars 100 μm. AFP in gill and skin samples was detected using antisera to skin type AFP. Normal rabbit serum was used as a control (Murray et al. 2002, 2003). (For color detail, please see the color plate section.)

combination with the high salt concentrations, the freezing point would be reduced below that of sea water (Wendelaar Bonga and Meij 1989; Evans et al. 2007).

LOW TEMPERATURE LIMITATIONS TO SEA CAGE AQUACULTURE

Atlantic salmon farming in the Maritimes, Canada, started in 1978 and became a commercial success in the coastal waters of Charlotte County, southwestern New Brunswick (Sylvia et al. 2000; Anderson 2007). This success created considerable interest in expanding the industry throughout Atlantic Canada, thus helping to improve the economic viability of many coastline communities. However, the extent of sea ice cover over the coastal waters off the north east coast of Atlantic Canada during the winter is a major impediment to the development of fish aquaculture (Fig. 16.6). This has restricted expansion of sea cage culture to the inshore regions along the south coast of Newfoundland, the southeast coast of New Brunswick, and the coast of Maine (USA) where the occurrence of sub-zero water temperatures and ice is rare. The value of the aquaculture industry in this region exceeds $100 million annually. However, even at these southerly locations, the danger that water temperatures may decline to lethal levels remains, and mortalities attributed to superchill are not uncommon (Aiken 1986; Page and Robinson 1992; Morgan 1993a, 1993b). An example of the ice conditions that can occur at a sea cage site during a severe winter is illustrated in Figure 16.7. The most recent case of superchill occurred during the winter of 2003 when sub-zero water temperatures extended as far south as Penobscot Bay, Maine, resulting in losses to the industry of $12 million (Raynor and Campbell 2003; Schreiber 2003).

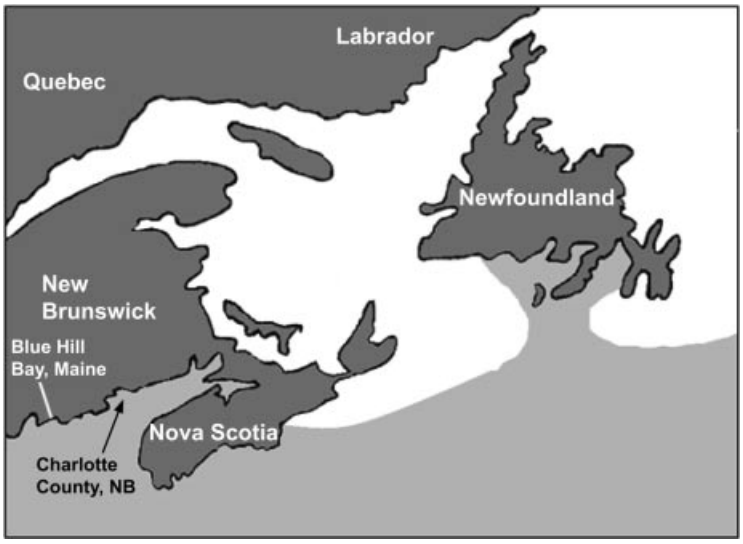


Figure 16.6. Maximum spring sea ice conditions in Atlantic Canada. The area in which sea ice can be encountered is indicated in white. (Adapted from Fletcher et al. 1992.)

When the aforementioned danger was brought to our attention in 1982, we were engaged in studying the antifreeze protein genes that were responsible for conferring freeze resistance to a variety of marine fish that inhabited icy waters. In addition, *Nature* had just published a front cover illustration of a growth hormone transgenic mouse, providing a dramatic demonstration that animal phenotypes could be altered by gene transfer (Palmiter et al. 1982). This success gave us reason to believe that salmon with increased freeze resistance could be generated if antifreeze protein genes were introduced into their genome.

ANTIFREEZE PROTEIN GENE TRANSFER; PROGRESS TO DATE

Thanks to strategic grant funds from the Natural Sciences and Engineering Research Council of Canada.

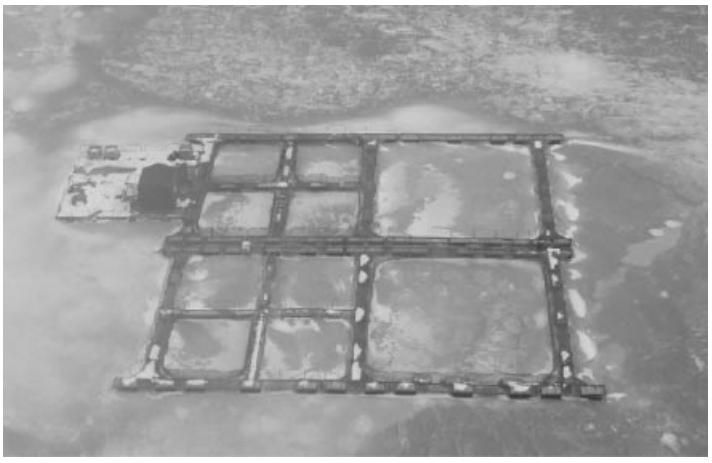


Figure 16.7. Ice conditions in and around an aquaculture sea cage operation during a severe winter. Photo courtesy of Jennifer Caines.

We (along with Professor Choy L. Hew) initiated our AFP gene transfer studies during the fall of 1983 using the 2a7 AFP gene that coded for one of the major small liver-specific AFP (HPLC 6) found in the blood of winter flounder. Initially, we spent a considerable amount of time and effort learning how to handle and microinject Atlantic salmon eggs and have them survive. This was also the period before PCR techniques came into use, thus the only reliable method for screening the microinjected fish for the presence of transgenes was through the use of Southern blotting procedures. Considering the fact that we generally injected thousands of fertilized eggs, this was a monumental task.

Our first evidence of success came with eggs injected in 1985 when we were able to demonstrate, using genomic Southern blotting, that antifreeze protein transgenes had integrated into the genome of 2 of the 30 fingerling Atlantic salmon that were screened (Fletcher et al. 1988). With the advent of PCR and use of Western blotting techniques, we were able to further demonstrate that the AFP transgene and its expression exhibited stable Mendelian inheritance over multiple generations (Shears et al. 1991; Hew et al. 1999). Details of this early research have been summarized and reviewed extensively elsewhere (Fletcher and Davies 1991; Fletcher et al. 1992; Hew et al. 1995; Fletcher et al. 2004).

The limitation of this research to date is that peak levels of AFP found in the plasma of the transgenic salmon (0.2–0.4 mg/mL) are well below the 10–15 mg/mL concentrations required to improve their freeze resistance (Hew et al. 1999). Similar results were obtained using a type III AFP gene (op5a) isolated from ocean pout (Hobbs and Fletcher 2008). Since type I and type III AFP are encoded by multigene families in their host fishes, it appears likely that the low levels of AFP found in the plasma of the transgenic salmon are attributable to low transgene copy number (Hew et al. 1988; Fletcher et al. 2001). If this is the case, then it is interesting to note that the level of AFP found in the plasma of the type I AFP transgenic salmon would be consistent with the expression of one of the 30–40 tandemly repeated liver-type AFP genes in winter flounder (Scott et al. 1985).

One very positive outcome of our research on AFP gene transfer research was that it provided the "proof of concept" that exogenous genes can be integrated into the salmon genome, expressed appropriately, and transferred from generation to generation in a predictable Mendelian fashion. This evidence laid the foundation for subsequent gene transfer studies in salmonids. A prime example of this is the successful production of a rapidly growing line of GH transgenic Atlantic salmon that has been patent protected and transferred to the private sector for commercial production (Hew and Fletcher 1996; Fletcher et al. 2004; Yaskowiak et al. 2006; Aqua Bounty Technologies, 2010).

FUTURE DIRECTIONS IN THE PRODUCTION OF FREEZE-RESISTANT SALMON

The molecular strategies employed by the winter flounder and other species to avoid freezing during winter serve as a clear blueprint delineating the requirements for making freeze-resistant fish. First and foremost, the AFP need to be secreted into the circulatory system and distributed throughout the extracellular space at levels that are sufficient to lower the freezing point down to that of the surrounding sea water. This can be accomplished by high AFP concentrations or lower concentrations of highly active AFP. The second point of importance is that AFP must be produced at appropriate concentrations in all external epithelia that come into contact with the surrounding environment. In the case of the skin epithelial cells, it is evident that AFP must be exported to the interstitial space, while in the gill epithelia, they should remain within the cytoplasm of the cells.

Increasing Functional Levels of AFP

Past research using type I and type III AFP genes has demonstrated that we need to find a means of increasing the levels of AFP activity within circulatory system and other extracellular fluids, and within the external epithelial cells. One possible way of accomplishing this is to increase the transgene dosage by the use of gene constructs containing multiple copies of tandem repeated AFP genes. If the winter flounder does require all of its 30–40 tandemly repeated AFP genes to be expressed in order to protect them from freezing, then it is going to be extremely

difficult to transfer a gene construct of such size. In addition, it has been well established that injected DNA sequences undergo considerable reorganization during embryogenesis. Such changes include rearrangements of the DNA sequence and concatemerization that results in multiple copy transgene integrants (Fletcher and Davies 1991; Wu et al. 2004; Uh et al. 2006; Yaskowiak et al. 2006; Butler and Fletcher 2009). Therefore, attempting to transfer multiple copy constructs is likely to exacerbate this problem. A further complication to this approach is the requirement by government regulatory agencies for a complete description of the transgene integrant, including elements of the bordering genomic DNA (Yaskowiak et al. 2006; Butler and Fletcher 2009). This is very difficult to accomplish using currently available techniques for nonmodel fish.

A second means to increasing the level of transgene expression is through the use of a strong promoter/enhancer such as that of the rainbow trout serum albumin gene (Hew et al. 1995). The combination of an albumin promoter linked to AFP coding sequences could result in increased plasma AFP levels. However, given the levels of small molecular weight type I and type III AFP required to protect the winter flounder and ocean pout from freezing, it is not certain that the increased expression would be sufficient to significantly improve the freeze resistance of the recipient fish.

In our view, the preferred approach to producing high functional levels of AFP is to develop a gene construct using a more powerful AFP. Some of the hyperactive AFP isolated from insects appears to be prime candidates for such a construct because they are 10–100 times more active than the small molecular weight type I and III AFP isolated from fish. A study of the in vivo efficacy of the spruce budworm AFP in rainbow trout confirmed this hypothesis by revealing that it was approximately 25 times more effective at improving their freeze resistance than was the small molecular weight type I AFP (Fig. 16.8). These results also suggest that if the level of expression of a spruce budworm AFP transgene in salmon matched that found for the type I AFP transgene, 0.2–0.4 mg/mL (∼60–120 μM), the functional antifreeze activity would be equivalent to 5–10 mg/mL of type I AFP (25 × 0.2–0.4 mg/mL).

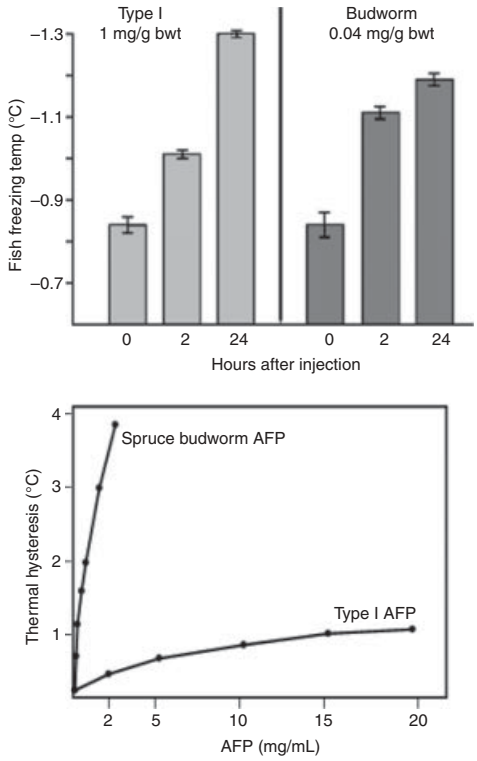


Figure 16.8. Efficacy of type 1 and spruce budworm (*Choristoneura fumiferana*) AFP at improving the freeze resistance of sea water-adapted rainbow trout. *Lower panel*: Liver type 1 and spruce budworm AFP activity (thermal hysteresis) as a function of AFP concentration. Thermal hysteresis data from Gong et al. (1996) and Tyshenko et al. (1997). *Upper panel*: The influence of AFP on the freeze resistance of rainbow trout. Rainbow trout were adapted to 80% sea water at a temperature of 0–1°C for several months. They were then injected intraperitonealy with liver type I (1 mg/g body weight) or spruce budworm (0.04 mg/g body weight) AFP and placed in a temperature-controlled water bath at −0.5°C where the temperature was lowered gradually by the addition of ice crystals. The lethal freezing temperature was established when the fish exhibited erratic swimming behavior (see Fletcher et al. 1986 for details). All values expressed as means ± SE. $N = 2$ for type I AFP and 4 for spruce budworm AFP experiments.

An alternative method of producing highly active AFP is through the use of protein engineering. There is considerable evidence from studies on natural AFP variants that the activity of an AF(G)P directly correlates with the molecular size of the protein (Schrag et al. 1982; Kao et al. 1986; Wu et al. 2001). Further research demonstrated that increasing the size of type III AFP (6 kDa) by creating fusion proteins with either thioredoxin (12 kDa) or maltose-binding protein (42 kDa) also enhanced activity (DeLuca et al. 1998). More recently, Marshall et al. (2004a) elaborated on this theme by examining the structural–functional relationship of the highly active β-helical AFP from the beetle *Tenebrio moliter*. The Tenebrio AFP consists of seven repetitive loops, each of which possesses an ice-binding motif. In order to examine the relationship between the area of the ice-binding surface and antifreeze activity, isoforms of this wild-type structure were engineered by the addition and removal of loops to create a range of constructs with 5–13 loops (Fig. 16.9). The results demonstrated that removal of a single coil dramatically reduced the antifreeze activity by approximately 80%, while the addition of two coils doubled the activity.

What is striking about the aforementioned results of AFP protein engineering is that a fish transgenic for the enhanced Tenebrio AFP would only require expression levels of 0.9 μM or 0.01 mg/mL in the blood plasma to enhance its freeze resistance by 2°C. This level of expression is considerably lower than that observed (60–120 μM) in our type I AFP transgenic salmon (Hew et al. 1999). Therefore, even if the enhanced Tenebrio transgene were driven by a weak fish AFP promoter, these levels of expression would be more than sufficient to protect the fish from freezing.

The downside to using a powerful insect AFP to create food for human consumption lies with public perception. A product containing this AFP may meet all of the food safety requirements mandated by government regulatory agencies, but fail at the level of public acceptance. Although education may eventually improve public acceptance of transgenic products containing nonfish or synthetic genes; at present, it seems unlikely that investors will risk the millions of dollars required to take such transgenic animal food products through the regulatory process.

A bright side to the possibility of producing freeze-resistant fish for aquaculture came with the

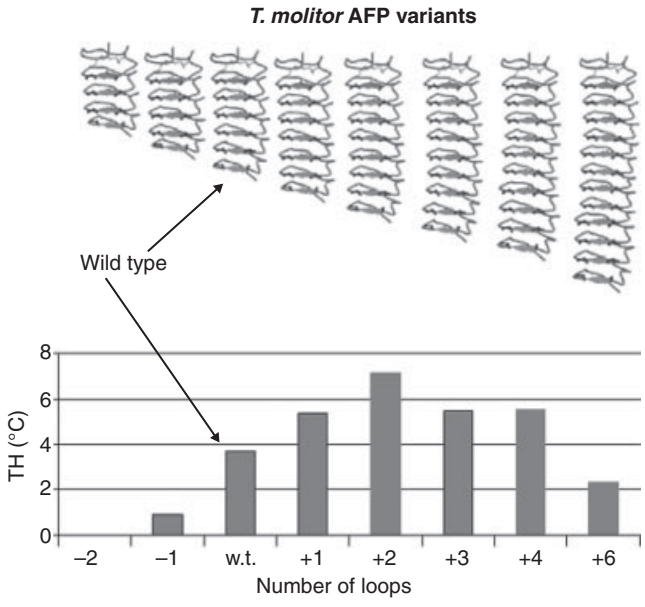


Figure 16.9. Protein engineering to enhance AFP activity. The wild-type *Tenebrio molitor* AFP possesses seven repetitive loops, each of which contains an ice-binding motif. A series of seven constructs were synthesized by subtracting loops from or adding loops to the seven coil parent AFP, and their activity (thermal hysteresis) is examined at a concentration of 60 μM (wild type = 0.7 mg/mL; Marshall et al. 2004a).

recent discovery of a hyperactive AFP from winter flounder (Marshall et al. 2004b, 2005; Fig. 16.2). Judging from its in vitro activity, this AFP is capable of improving the freeze resistance of fish by 2°C at a plasma concentration of 0.3 mg/mL (17 μM), a level of expression comparable to that observed in type I transgenic salmon where there was no improvement in their freeze resistance (Hew et al. 1999).

Tissue-Specific Expression

Making transgenic fish that can express and export AFP from the liver and from the skin epithelial cells while retaining the AFP within the cytoplasm of the gill epithelia will likely be challenging. One approach would be to build a transgene that could be expressed and the AFP exported by the cells of all tissues, including liver and external epithelia. Whether or not the AFP would function to protect gill epithelia remains to be established. In addition, the possible pleiotropic effects of AFP expression in all cells of the body are unknown.

A second, less attractive approach would be to develop three different constructs: one that possesses a secretory signal and liver-specific regulatory elements, a second construct with the secretory signal that could be developed using skin-specific elements, and a third skin-specific construct that could be designed without the signal sequence. One difficulty with this approach would be getting all three transgenes integrated into the same chromosome so that they can be inherited by subsequent generations in a stable and predictable manner. This difficulty could perhaps be overcome by linking all three constructs together prior to injection. However, such a chimeric construct could be as much as 12 kb in length, thus increasing its susceptibility to fragmentation and reorganization prior to genomic integration (Fletcher and Davies 1991). Some of these difficulties may eventually be resolved by the use of transposable elements such as To12, which has been shown to be highly successful in zebrafish (MacDonald and Ekker, Chapter 15).

SUMMARY AND CONCLUSIONS

It seems clear that the road to follow in the production of more freeze-resistant fish for aquaculture is to use AFP that are more powerful than the small type I and type III AFP used in past attempts. The discovery of hyperactive AFP in insects and the engineering of AFP with improved activity were major breakthroughs in this regard. However, they may not be acceptable for the production of transgenic fish for human consumption at the present time. Nevertheless, they do have considerable potential value for biotechnological applications in cryobiology and in products such as nonfood organisms and ornamental plants (Fletcher et al. 1999). This leaves the hyperactive AFP found in winter flounder as the AFP of choice in the production of freeze-resistant transgenic food fish. This protein, expressed in low concentrations, is quite capable of protecting fish from freezing in ice-laden sea water. The question that remains to be resolved concerns the design of gene constructs that will enable the AFP to be expressed and function appropriately in liver and external epithelia.

In our view, the production of freeze-resistant fish by duplicating the strategies employed by freeze-resistant species such as the winter flounder is likely to be successful in the long term, and the outcome could be of considerable value to cold water aquaculture. However, finding the means of employing these strategies while addressing the requirements of government regulatory agencies with regards to food safety is no small task (Fletcher et al. 2004; Canadian Food Inspection Agency 2008; Fox 2008; FDA 2009).

REFERENCES

Anderson JM. 2007. The Salmon Connection. Glen Margaret Publishing, Tantallon, NS Canada. pp 28–30.

Aiken D. 1986. Chill waters test mettle of Fundy salmon farmers. Canadian Aquaculture. 2: 27–29.

Aqua Bounty Technologies. 2010. http://www.aquabounty.com/

Baardness J and Davies PL. 2001. Sialic acid synthase: the origin of type III antifreeze protein? Trends Biochem Sci. 26: 468–469.

Bartels H and Potter IC. 1993. Intercellular junctions in the water–blood barrier of the gill lamella in the adult lamprey (*Geotria australis, Lampetra fluviatilis*). Cell Tissue Res. 274: 521–532.

Bullock AM and Roberts RJ. 1974. The dermatology of marine teleost fish. I. The normal integument. Oceanogr Mar Biol A Rev. 13: 383–411.

Burton D and Fletcher GL. 1983. Seasonal changes in the epidermis of the winter flounder, *Pseudopleuronectes americanus*. J Mar Biol Ass UK. 63: 273–287.

Butler TM and Fletcher GL. 2009. Promoter analysis of a growth hormone transgene in Atlantic salmon. Theriogenology. 72: 62–71.

Canadian Food Inspection Agency. 2008. Notification guidelines for the environmental assessment of the use of animal biotechnology in livestock. http://www.inspection.gc.ca/english/anima/biotech/guidedirecte.shtml.

Chen L, DeVries AL, and Cheng CH. 1997. Convergent evolution of antifreeze glycoproteins in Antarctic notothenioid fish and Arctic cod. Proc Natl Acad Sci USA. 94: 3817–3822.

Davies PL, Fletcher GL, and Hew CL. 1989. Fish antifreeze protein genes and their use in transgenic studies. In: Oxford Surveys on Eukaryotic Genes (ed. Maclean N). Oxford University Press, New York. pp 85–109.

Davies PL, Fletcher GL, and Hew CL. 1999. Freeze-resistance strategies based on antifreeze proteins. In: Environmental Stress and Gene Gegulation (ed. Storey KB). Bios Scientific Publishers, Oxford, UK. pp 61–80.

DeLuca CI, Comley R, and Davies PL. 1998. Antifreeze proteins bind independently to ice. Biophys J. 74: 1502–1508.

Deng G, Andrews DW, and Laursen RA. 1997. Amino acid sequence of a new type of antifreeze protein, from the longhorn sculpin *Myoxocephalus octodecimspinosis*. FEBS Lett. 402: 17–20.

Desjardins M, Le Francois NR, Fletcher GL, and Blier PU. 2006. Seasonal modulation of plasma antifreeze protein levels in Atlantic (*Anarhichas lupus*) and spotted (*A. minor*) wolffish. J Exp Mar Biol Ecol. 335: 142–150.

DeVries AL, Komatsu SK, and Feeney RE. 1970. Chemical and physical properties of freezing-point depression glycoproteins from Antarctic fishes. J Biol Chem. 245: 2901–2913.

Evans RP and Fletcher GL. 2001. Isolation and characterization of type I antifreeze proteins from Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*Liparis gibbus*). Biochimica et Biophysica Acta. 1547: 235–247.

Evans RP and Fletcher GL. 2004. Isolation and characterization of antifreeze proteins from skin tissues of snailfish, cunner and sea raven. Biochim Biophys Acta. 1700: 209–217.

Evans RP and Fletcher GL. 2005a. Type 1 AFPs expressed in skin tissues of Atlantic (*Liparis atlanticus*) and dusky (*Liparis gibbus*) snailfish are identical to their circulating plasma proteins. FEBS J. 272: 5327–5336.

Evans RP and Fletcher GL. (2005b). Molecular cloning of liver expressed proteins from Atlantic snailfish: possible evolutionary origins of type I AFPs. J Mol Evol. 61: 417–424.

Evans RP, Hobbs RS, Goddard SV, and Fletcher GL. 2007. The importance of dissolved salts to the in vivo efficacy of antifreeze proteins. Comp Biochem Physiol A. 148: 556–561.

Ewart KV and Fletcher GL. 1990. Isolation and characterization of antifreeze proteins from smelt (Osmerus mordax) and Atlantic herring (*Clupea harengus harengus*). Can J Zool. 68: 1652–1658.

FDA. 2009. Genetically engineered animals. http://www.fda.gov/cvm/GEAnimals.htm

Fletcher GL and Davies PL. 1991. Transgenic fish for aquaculture. In Genetic Engineering, Principles and Methods (ed. Setlow J). Plenum Press, New York. Vol 13. pp 331–370.

Fletcher GL, Davies PL, and Hew CL. 1992. Genetic engineering of freeze-resistant Atlantic salmon. In: Transgenic Fish (eds. Hew CL and Fletcher GL). World Scientific Publishing Co, Singapore. pp 190–208.

Fletcher GL, Goddard SV, and Wu Y. 1999. Antifreeze proteins and their genes: from basic research to business opportunity. Chemtech. 29: 17–28.

Fletcher GL, Hew CL, and Davies PL. 2001. Antifreeze proteins of teleost fishes. Annu Rev Physiol. 63: 359–390.

Fletcher GL, Kao MH, and Fourney RM. 1986. Antifreeze peptides confer freezing resistance to fish. Can J Zool. 64: 1897–1901.

Fletcher GL, King MJ, Kao MH, and Shears MA. 1989. Antifreeze proteins in the urine of marine fish. Fish Physiol Biochem. 6: 121–127.

Fletcher GL, Shears MA, King MJ, Davies PL, and Hew CL. 1988. Evidence for antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). Can J Fish Aquat Sci. 45: 352–357.

Fletcher GL, Shears MA, Yaskowiak E, King MJ, and Goddard SV. 2004. Gene transfer: potential to enhance the genome of Atlantic salmon for aquaculture. Aust J Exp Ag. 44: 1095–1100.

Fox JL. 2008. FDA transgenic animal guidance finally surfaces. Nat Biotechnol. 26: 1205–1207.

Gauthier SY, Scotter AJ, Lin FH, Baardsnes J, Fletcher GL, and Davies PL. 2008. A re-evaluation of the role of type IV antifreeze protein. Cryobiology. 57: 292–296.

Gong Z, Fletcher GL, and Hew CL. 1992. Tissue distribution of fish antifreeze protein mRNAs. Can J Zool. 70: 810–814.

Gong Z, Hu Z, Ewart KV, Fletcher GL, and Hew CL. 1996. Skin antifreeze protein genes of the winter flounder encode distinct and active polypeptides without the secretory signal sequences. J Biol Chem. 271: 4106–4112.

Graham LA, Lougheed SC, Ewart KV, and Davies PL. 2008a. Lateral transfer of a lectin-like antifreeze protein gene in fishes. PLoS ONE. 3: e2616.

Graham LA, Marshall CB, Lin F-H, Campbell RL, and Davies PL. 2008b. Hyperactive antifreeze protein from fish contains multiple binding sites. Biochem. 47: 2051–2063.

Hew CL and Fletcher GL. 1996. Transgenic salmonid fish expressing exogenous salmonid growth hormone. United States Patent No. 5545808.

Hew CL, Fletcher GL, and Davies PL. 1995. Transgenic salmon: tailoring the genome for food production. J Fish Biol. 47(Suppl A): 1–19.

Hew CL, Poon R, Xiong F, Gauthier S, Shears MA, King MJ, Davies PL, and Fletcher GL. 1999. Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. Transgenic Res. 8: 405–414.

Hew CL, Wang NC, Joshi S, Fletcher GL, Scott GK, Hayes PH, Buettner B, and Davies PL. 1988. Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, *Macrozoarces americanus*. J Biol Chem. 263: 12049–12055.

Hobbs RS and Fletcher GL. 2008. Tissue-specific expression of antifreeze protein and growth hormone transgenes driven by the ocean pout (*Macrozoarces americanus*) antifreeze protein OP5a gene promoter in Atlantic salmon (Salmo salar). Transgenic Res. 17: 33–45.

Kao MH, Fletcher GL, Wang NC, and Hew CL. 1986. The relationship between molecular weight and antifreeze polypeptide activity in marine fish. Can J Zool. 64: 578–582.

Larese A, Acker J, Muldrew K, Yang H, and McGann L. 1996. Antifreeze proteins induce intracellular nucleation. Cryo-Letters. 17: 175–182.

Marshall CB, Chakrabartty A, and Davies PL. 2005. Hyperactive antifreeze protein from winter flounder is a very long rod-like dimer of α-helices. J Biol Chem. 280: 17920–17929.

Marshall CB, Daley ME, Sykes BD, and Davies PL. 2004a. Enhancing the activity of a β-helical antifreeze protein by the engineered addition of coils. Biochem. 43: 11637–11646.

Marshall CB, Fletcher GL, and Davies PL. 2004b. Hyperactive antifreeze protein in a fish. Nature. 429: 153.

Morgan S. 1993a. New Brunswick loses fish to cold temperatures, moving cages helps. Atlantic Fish Farming. 27: 3.

Morgan S. 1993b. Water temps in slow decline. Atlantic Fish Farming. 27: 5.

Murray HM, Hew CL, and Fletcher GL. 2003. Skin-type antifreeze protein expression in integumental cells of larval winter flounder (*Pleuronectes americanus*). J Fish Biol. 60: 1391–1406.

Murray HM, Hew CL, Kao KR, and Fletcher GL. 2002. Localization of cells from the winter flounder gill expressing a skin-type antifreeze protein gene. Can J Zool. 80: 110–119.

Nishimiya Y, Sato R, Takamichi M, Miura A, and Tsuda S. 2005. Co-operative effect of the isoforms of type III antifreeze protein expressed in Notched-fin eelpout, Zoarces elongatus Kner. FEBS J. 272: 482–492.

Page F and Robinson S. 1992. Salmon farming in the Bay of Fundy: a chilling reminder. World Aquacult. 23: 31–34.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, and Evans RM. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein–growth hormone fusion genes. Nature. 300: 611–615.

Pertaya N, Marshall CB, DiPrinzio CL, Wilen L, Thomson ES, Wettlaufer JS, Davies PL, and Braslavsky I. 2007. Fluorescence microscopy evidence for quasi-permanent attachment of antifreeze proteins to ice surfaces. Biophys J. 92: 3663–3673.

Pickett MH, Scott G, Davies P, Wang N, Joshi S, and Hew C. 1984. Sequence of an antifreeze protein precursor. Eur J Biochem. 143: 35–38.

Raynor B and Campbell M. 2003. Superchill hits maritimes hard. Fish Farming. 15: 1–2.

Raymond JA and DeVries AL. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc Nat Acad Sci USA. 82: 2589–2593.

Sandbacka M, Lilius H, Enkvist MOK, and Isomas B. 1998. Rainbow trout gill epithelial cells in primary culture communicate through gap junctions

as demonstrated by dye coupling. Fish Physiol Biochem. 19: 287–292.

Schreiber L. 2003. Swans Island—cultured salmon have been having a hard time with the past month's arctic temperatures. http://www.growfish.com.au/grow/pages/news/2003/mar2003/582103.htm

Scott GK, Hew CL, and Davies PL. 1985. Antifreeze protein genes are tandemly linked and clustered in the genome of the winter flounder. Proc Natl Acad Sci USA. 82: 2613–2617.

Scott B and Scott MG. 1988. Atlantic fishes of Canada. Can Bull Fish Aquat Sci. 219: 554–557.

Schrag JD, O'Grady SM, and DeVries AL. 1982. Relationship of amino acid composition and molecular weight of antifreeze glycopeptides to non-colligative freezing point depression. Biochim Biophys Acta. 717: 322–326.

Shears MA, Fletcher GL, Hew CL, Gauthier S, and Davies PL. 1991. Transfer, expression and inheritance of antifreeze protein genes in Atlantic salmon (Salmo salar). Mol Mar Biol Biotechnol. 1: 58–63.

Shears MA, Kao MH, Scott GK, Davies PL, and Fletcher GL. (1993). Distribution of type III antifreeze proteins in Zoarcoidei. Mol Mar Biol Biotechnol. 2: 104–111.

Sylvia G, Anderson GL, and Hanson E. 2000. The new order in global salmon markets and aquaculture development: implications for watershed-based management in the Pacific Northwest. In: Sustainable Fisheries Management: Pacific Salmon (eds. Knudsen EE, Steward CR, MacDonald DD, Williams JE, and Reiser DW). CRC Press, New York. pp 393–405.

Tyshenko MG, Doucet D, Davies PL, and Walker VK. 1997. The antifreeze potential of the spruce budworm thermal hysteresis protein. Nat Biotech. 15: 887–890.

Uh M, Kttra A, and Devlin RH. 2006. Transgene constructs in coho salmon (*Oncorhynchus kisutch*) are repeated in a head-to-tail fashion and can be integrated adjacent to horizontally-transmitted parasite DNA. Transgenic Res. 15: 711–727.

Valerio PF, Kao MH, and Fletcher GL. 1992. Fish skin: an effective barrier to ice crystal propagation. J Exp Biol. 164: 135–151.

Wendelaar Bonga SF and Meij CJM. 1989. Degeneration and death, by apoptosis and necrosis, of the pavement and chloride cells in the gills of the teleost *Oreochromis massambicus*. Cell Tissue Res. 255: 235–243.

Wu B, Sun Y-H, Wang Y-P, Wang Y-W, and Zhu Z-Y. 2004. Sequences of transgene insertion sites in transgenic F4 common carp. Transgenic Res. 13: 95–96.

Wu Y, Kao MH, Goddard SV, Banoub J, and Fletcher GL. 2001. Antifreeze glycoproteins: relationship between molecular weight, thermal hysteresis and the inhibition of leakage from liposomes during thermotropic phase transition. Comp Biochem Physiol B. 128: 265–273.

Yaskowiak ES, Shears MA, Agarwal-Mawal A, and Fletcher GL. 2006. Characterization and multigenerational-stability of the growth hormone transgene (EO-1α) responsible for enhanced growth rates in Atlantic salmon. Transgenic Res. 15: 465–480.

Young HM and Fletcher GL. 2008. Antifreeze protein gene expression in winter flounder pre-hatch embryos: implications for cryopreservation. Cryobiology. 57: 84–90.

Chapter 17

Potential Applications of Transgenic Fish to Environmental Monitoring and Toxicology

Hwee Boon Grace Ng, Siew Hong Lam, Hendrian Sukardi, and Zhiyuan Gong

INTRODUCTION

Transgenic technology has been widely used in biotechnology from the generation of genetically modified food to the production of pharmaceutical proteins. Since it was first applied to fish in the mid-1980s, transgenic studies have been carried out in over 35 fish species, half of which are important for aquaculture (Zbikowska 2003). Most of the early transgenic fish studies focused on generation of superior aquaculture fish stocks with beneficial traits such as enhanced growth rate, freezing resistance, or disease resistance (Nam et al. 2007). Despite the success of producing genetically modified fish stock, marketing of transgenic fish as food remains a controversial issue due to ecological and food safety concerns. So far, the only successful case of commercializing transgenic fish is the GloFish, transgenic zebrafish that expressed fluorescent proteins for ornamental display (Gong et al. 2003; Knight 2003).

Nevertheless, the transgenic fish approach in basic research, particularly by using tissue-specific promoters and living color reporter gene (e.g., GFP or green fluorescent protein), has become a popular experimental tool in developmental analysis as well as for characterization of gene promoters (Gong et al. 2001, 2004; Udvadia and Linney 2003). A potential application that arises from the current knowledge of gene regulation is to engineer biomonitoring transgenic fish. This is to develop transgenic fish by using gene constructs comprising a pollutant-inducible promoter to drive a reporter gene; expression of the reporter gene would indicate the presence of certain environmental pollutants. If the reporter gene encodes a living color protein such as GFP, such biomonitoring transgenic fish would display visible color change when they are exposed to polluted water, hence providing a convenient biomonitoring system. In the past few years, we and several other groups have generated a few lines of transgenic

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

fish for environmental monitoring. In this communication, we summarize the current progress in this area.

SMALL FISH MODELS AS SENTINELS IN AQUATIC TOXICOLOGY: ZEBRAFISH AND MEDAKA

The zebrafish (*Danio rerio*) and the medaka (*Oryzias latipes*) have emerged as dominant models in the development of biomonitoring transgenic fish because of the ease of genetic analyses and the established transgenic technology in these two small freshwater fish species. The zebrafish is a tropical freshwater fish that belongs to the family Cyprinidae. Its natural habitats are found in rivers of South Asia, mainly northern India as well as northern Pakistan, Bhutan, and Nepal (Dahm and Geisler 2006). The medaka, a freshwater fish that belongs to the family Adrianichthyidae, is native to Asia (primarily Japan, Korea, and eastern China) (Wittbrodt et al. 2002). Both zebrafish and medaka are popular in genetic and developmental studies because of the common attributes such as short generation time, high fecundity, ease of manipulation, and microinjection of transparent embryos as well as ease of fish husbandry. Moreover, the availability of vast genomic resources to the scientific community also fuelled the work carried out on these two fish species (UTGB Medaka; Kobayashi and Takeda 2008; Sanger 2009).

It is also worth noting that the medaka provides additional advantages in the development of biomonitoring fish compared to the zebrafish. Firstly, the medaka is a temperate fish and is able to inhabit a wide range of temperatures from near 0°C to 30°C, while the zebrafish, as a tropical species, has a narrow temperature requirement from 24°C to 30°C. Secondly, the medaka is also able to adapt to a broader range of salinity than zebrafish and most other freshwater fish. Thirdly, the sex of medaka can be identified both morphologically and genetically, hence providing added advantages when monitoring certain contaminations that influence sex and reproduction. Fourthly, the medaka has a longer history in toxicological studies and abundant information has been accumulated for further investigation in ecotoxicology. Lastly, the body wall of the laboratory medaka strain is more transparent; hence, GFP expression in internal organs can be detected more sensitively in medaka than zebrafish. In particular, a completely transparent strain of medaka, *see-through*, is available and internal organs including the brain can be observed directly by the naked eyes (Wakamatsu et al. 2001).

MODELS OF BIOMONITORING FISH

The concept of developing transgenic biomonitoring fish is largely based on the availability of inducible gene promoters and living color reporter genes. Since the development of the first GFP transgenic fish in 1995 (Amsterdam et al. 1995), there has been a rapid increase in living color transgenic fish lines for various purposes, such as gene expression patterns, tissue/organ development, cell lineage tracing, and promoter/enhancer trap (Gong et al. 2001, 2004; Udvadia and Linney 2003). Most of the transgenic models engineered for ecotoxicology used fluorescent protein as the reporter, especially the enhanced GFP from Clontech, due to its ease of detection. Classic reporter genes such as *CAT* (*chloramphenicol acetyltransferase*), *LacZ* (*β-galactosidase*), and *luciferase* have limitations as they often require sacrifice of the fish for laborious enzymatic assays, thus hampering multi-time point analysis on a single subject. In comparison, the living color proteins can be directly visualized in vivo without the need of adding exogenous substrates or cofactors for detection. They, therefore, allow for dynamic real-time observation of the expression patterns for longer periods in live embryos as well as whole organisms.

The fluorescence intensity of GFP expression is difficult to quantify, hence the *luciferase* gene is often used as an alternative to quantify transgene activity (Mattingly et al. 2001; Kusik et al. 2008). Even so, relative GFP quantification has been successfully performed by modifying and optimizing the detection system. Seok et al. (2007) have proposed a fluorescence-based protein quantification system with the underlying assumption that fluorescence intensity of purified GFP is directly proportional to the protein quantity. Soluble proteins are extracted from the transgenic zebrafish embryos and measured for relative fluorescence using PerkinElmer Victor^3 1420 multilabel counter, and the readings are then extrapolated from the standard curve of fluorescence intensity derived from serially diluted GFP of

known concentrations. In another study, Kurauchi et al. (2005) used a cooled charge-coupled device (CCD) camera to determine the exposure time of a fluorescent tissue in order to obtain a constant fluorescence intensity. Likewise, the amount of GFP in the tissue was extrapolated from a standard curve of reciprocal exposure time measured from known GFP concentrations.

Here, we briefly describe various transgenic fish models according to the categories of targeted pollutants. Table 17.1 summarizes some of these transgenic models.

Estrogenic Compounds

Estrogenic substances are a major environmental pollution concern because they can cause endocrine disruptions such as reproductive impairment of wildlife and humans (Matozzo et al. 2008). One of the most reported effects of xenoestrogens is the induction of vitellogenins (Vtgs), precursors of the egg yolk proteins, which are produced under the control of the estrogen receptor pathway in egg-laying vertebrates. Vtgs are normally synthesized in the liver of sexually mature females in response to endogenous estrogens. The Vtg level is usually undetectable or low in males and juveniles, but will be upregulated in response to estrogens or estrogen mimics. Since Vtgs first enter the blood for transportation to the oocytes, measurement of plasma Vtg levels has become one of the most useful indicators of estrogenic pollution (Matozzo et al. 2008).

Tong et al. (2004) has shown that *vtg* mRNAs are inducible in both zebrafish and medaka by exogenous 17-β-estradiol (E2). It appears that the medaka is more sensitive than the zebrafish to respond to E2, which has prompted our laboratory to develop a GFP transgenic medaka under the estrogen-inducible promoter from the medaka *vtg1* gene, *Tg(mvtg1:gfp)* (Zeng et al. 2005). Concomitant with endogenous vitellogenin production, the sexually mature female medaka of this transgenic line express GFP in the liver; however, no GFP expression is observed in the male counterpart (Fig. 17.1A). Yet, when induced with E2, the males express GFP specifically in the liver (Fig. 17.1B). Dose-dependent increases in GFP signal have also been observed in the livers of treated male fish. The LOEC (lowest-observed-effects concentration) of E2 for hemizygous transgenic fish is 0.1 μg/L and can be improved up to 0.05 μg/L for homozygous transgenic fish. This sensitivity is within the range of the E2 concentrations of 0.027–0.24 μg/L in sewage effluent that was estimated from vitellogenin induction studies in catfish and bass (Thompson et al. 2000). Several other estrogenic chemicals including 17-α-ethynylestradiol, estriol, diethylstilbestrol, bisphenol A, and 4-*n*-nonylphenol have also been shown to induce GFP expression in *vtg1:gfp* transgenic medaka although the extent of GFP induction differs with each chemical (Zeng et al. 2005). These authors also demonstrated that this transgenic line can be used repeatedly in monitoring E2. After the removal of E2 treatment, the GFP fluorescence of transgenic males decreases gradually and becomes undetectable by about one month. However, a second treatment with E2 on the same fish induces the GFP expression again and it seems that these fish become more sensitive in response to the second E2 treatment as the average GFP expression is above that of first treatment (Fig. 17.1C).

Estrogen-inducible GFP expression have also been demonstrated in several other GFP transgenic medaka lines using the medaka *choriogenin L* promoter (Salam et al. 2008; Ueno et al. 2004) and medaka *choriogenin H* promoter (Kurauchi et al. 2005). Both choriogenin H and L are precursors of egg envelope proteins and are produced in response to estrogens, hence GFP is expressed only in sexually mature transgenic females. Similar to our *Tg(mvtg1:gfp)* medaka, GFP expression is also induced in the liver of E2-treated males in these transgenic lines. Salam et al. (2008) have reported that their minimum detectable concentration of E2 was 0.025 μg/L, while Kurauchi et al. (2005) stated that their studies indicated a minimum level of 0.171 μg/L. Considering the LOEC of 0.1 μg/L in our *Tg(mvtg1:gfp)* transgenic line, the detection limits are similar in the three transgenic systems despite their differences and the fact that each laboratory has used its own arbitrary system of quantifying GFP expression. In addition, a zebrafish transgenic line with an artificial promoter and three tandem repeats of estrogen response element motifs linked to a luciferase gene has been developed by Leger et al. (2000). As expected, the luciferase activity is high in untreated adult females and can be induced in

P1: SFK/UKS
BLBS089-c17
P2: SFK
BLBS089-Fletcher
October 3, 2011
11:26
Trim: 246mm X 189mm
Printer Name: Yet to Come

Table 17.1. Examples of Biomonitoring Transgenic Fish Systems.

Categories of Pollutants	Gene Promoters	Species of Promoter	Reporter Genes	Fish Models	Systems	References
Estrogenic compounds	Tandem repeats of ERE	Artificial	*Luciferase*	Zebrafish	Stable	Legler et al. 2000
Estrogenic compounds	*Choriogenin L*	Medaka	*GFP*	Medaka	Stable	Ueno et al. 2004; Salam et al. 2008
Estrogenic compounds	*Vitellogenin 1*	Medaka	*GFP*	Medaka	Stable	Zeng et al. 2005
Estrogenic compounds	*Choriogenin H*	Medaka	*GFP*	Medaka	Stable	Kurauchi et al. 2005
Heavy metals	*Heat shock protein 70*	Zebrafish	*GFP*	Zebrafish	Stable	Blechinger et al. 2002
Heavy metals	*Heat shock protein 70*	Human	*GFP*	Zebrafish	Transient	Seok et al. 2006; Seok et al. 2007
Heavy metals	*Glutathione S-transferase α1*	Mouse	*Luciferase–GFP*	Zebrafish	Stable	Kusik et al. 2008
Heavy metals	*Heat shock protein 27*	Zebrafish	*GFP*	Zebrafish	Stable	Wu et al. 2008
Heavy metals and POPs	Various promoters with AHRE, EPRE, or MRE	Mouse or trout	*Luciferase*	Zebrafish cell culture	Transient	Carvan et al. 2000a
Xenobiotic compounds	*Cytochrome p450 1a1*	Human	*GFP, Luciferase*	Zebrafish and zebrafish cell culture	Transient	Mattingly et al. 2001
Xenobiotic compounds	*Cytochrome p450 1a1*	Human	*GFP*	Zebrafish cell culture	Transient	Seok et al. 2008
Mutagens	–	–	*rpsL*	Zebrafish	Stable	Amanuma et al. 2000
Mutagens	–	–	*cII, lacI*	Medaka	Stable	Winn et al. 2000, 2001
Mutagens	–	–	*lacZ*	Medaka, Mummichog	Stable	Winn et al. 2001

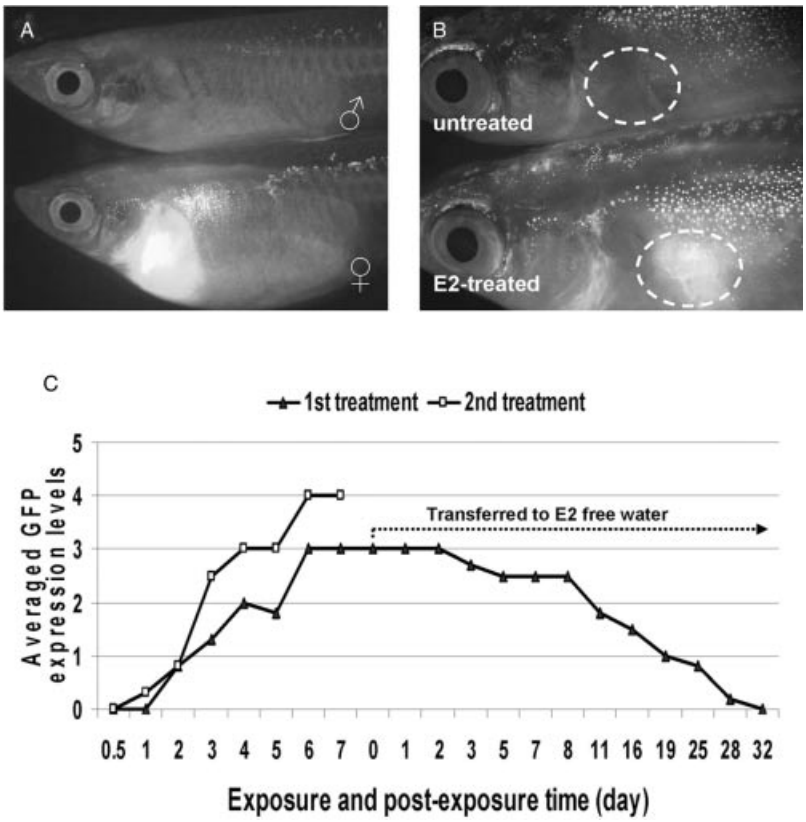


Figure 17.1. *Tg(mvtg1:gfp)* transgenic medaka. (**A**) Live adult male (top) and female (bottom) transgenic fish. Note that GFP expression is only in the liver of female transgenic fish. (**B**) Induction of GFP expression by exogenous 17β-estradiol (E2). Transgenic male fish were immersion-treated with 1 μg/L E2 for more than 12 hours and GFP expression was observed in the liver (bottom), while no GFP expression was observed in the liver area (circled) in untreated transgenic male fish (top). (**C**) Time course of GFP induction in transgenic male fish. Ten transgenic male fish were treated with 5 μg/L E2 for 7 days and GFP expression was induced to a relatively high level by 1 week. Thereafter, all of the fish were transferred into E2-free water on day 8 (or day 0 for E2-free water). GFP expression gradually decreased and was completely invisible by day 32 (see the 1st treatment curve with closed triangles). These fish were kept in estrogen-free water for another week and then treated with a second dose of E2 at the same concentration and GFP expression was reinduced (see the 2nd treatment curve with open squares). The details of the experiments and the estimation of relative levels of GFP expression have been described in our original publication (Zeng et al. 2005). Pictures and data are modified from Zeng et al. (2005). (For color detail, please see the color plate section.)

E2-treated males. Interestingly, Leger et al. (2000) have reported that the organs expressing the highest luciferase activity in E2-treated males were the testes, followed by the liver, suggesting the high sensitivity of gonads to environmental estrogens.

Heavy Metals

Heat shock proteins (HSPs) are a family of highly conserved molecular chaperones that are known to play a pivotal role in cell protection and repair upon stress. HSP70, the best characterized subfamily of

HSPs, is upregulated when cells are under stress from environmental and physiological duress. Numerous studies have shown that heavy metal treatments other than thermal shock and hypoxia elevated HSP70 levels, thus making it a potential marker in toxicology screening (Ireland et al. 2004; La Porte 2005). Blechinger et al. (2002) have used a GFP transgenic zebrafish line under a zebrafish *hsp70* promoter to detect heavy metals and found that GFP was first observed in the olfactory epithelium, gills, and skin when the stable transgenic fish were treated with cadmium. Seok et al. (2006, 2007) also used a transient transgenic zebrafish system to test copper and arsenite and found that most of the same tissues were sensitive to metal induction of GFP expression. Thus, it is likely that these tissues were the first to be directly exposed to waterborne metal ions. Taken together, these reports suggest that the *hsp70* promoter gene construct is useful for detecting heavy metal pollutants.

We have also generated a GFP transgenic zebrafish line using a small heat shock protein gene promoter (*hsp27*) and found that GFP expression can be induced by sodium arsenate but not by other metals such as cadmium, zinc, and mercury (Wu et al. 2008). Thus, this transgenic line may be useful to detect specific categories of chemicals.

Recently, another line of transgenic zebrafish has been described to respond to mercury treatment (Kusik et al. 2008). In this line, the reporter gene is under the regulation of the electrophile responsive element (EPRE) that responds to oxidative stressors such as heavy metals. The construct consisted of the EPRE sequence from the mouse *Gsta1* gene fused with a minimal promoter from the mouse *mt1* gene. A fusion reporter gene encoding a luciferase-GFP fusion protein was used for both visual detection and quantitative assessment. Although transient fish systems have shown both luciferase activity and GFP expression when treated with mercury, only luciferase activity has been observed in stable lines of transgenic zebrafish.

Persistent Organic Pollutants

Persistent organic pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate through the food chain, and pose a risk of causing adverse effects to human health and the environment (Weber et al. 2008). These compounds include polycyclic aromatic hydrocarbons (PAHs), dioxins, polyhalogenated biphenyls, dibenzofurans, and many of the pesticides. The cytochrome P450 family is a superfamily of hemoproteins that catalyze monooxgenase reactions essential for xenobiotic metabolism, and many of its members, including CYP1a1, are highly inducible by POPs. Mattingly et al. (2001) has constructed two reporter plasmids, p1a1Luc and p1a1GFP, with the promoter region from human cytochrome P450 regulatory regions linked to reporter genes, *luciferase* and *GFP*, respectively. Transfection of p1a1Luc into cultured zebrafish liver cells showed dose-dependent induction of luciferase activity when treated with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Transient p1a1GFP zebrafish embryos produced GFP expression after exposure to TCDD in the eyes, nose, and along the vertebrae, demonstrating the possibility of using the *cyp1a1* promoter to develop transgenic fish for detecting POPs.

Mutagens

Many of the POPs and heterocyclic amines are mutagenic and widespread and, therefore, pose significant health hazards to humans and other exposed vertebrates (Weber et al. 2008). Transgenic fish have been developed to perform mutation assays to characterize the DNA damage caused by pollutants in aquatic environments (Amanuma et al. 2000; Winn et al. 2000, 2001). Winn et al. (2000) have developed a λ transgenic medaka line that carries multiple copies of the λ bacteriophage vector where it contains either *lacI* or *cII* gene as neutral mutational targets. After exposure to mutagenic chemicals, the vector was extracted from the genomic DNA of transgenic fish and transferred into bacterial host cells for mutation analyses. Mutation frequencies as well as mutational spectra were determined. Since mutational spectra are consistent with known modes of mutagen action, various mutational profiles can be used to deduce the classes of pollutants. The approach is similar to other mutagenic transgenic fish lines that use plasmid vectors. Amanuma et al. (2000) have produced zebrafish harboring the pML4 plasmid vector fused to the *rspL* gene, while Winn et al. (2001) have produced transgenic medaka as well as mummichogs containing

the plasmid pUR288 vector with the *lacZ* as a mutational target.

TOXICITY SCREENING OF COMPOUNDS USING FLUORESCENT TRANSGENIC EMBRYOS AND LARVAE

The availability of a large number of transgenic fish lines expressing fluorescent proteins in specific or multiple tissues/organs (e.g., nervous system, vascular system, muscle, skin, liver, pancreas, and intestine) that have been produced for developmental studies can further be exploited for screening of compounds that cause developmental toxicity in these tissues or organs. The living color protein fluorescing in a specific tissue or organ of the transparent embryos can easily be visualized in real-time, and the development of the tissues or organs can be followed continuously in the same individual for assessment of various toxicological endpoints. For example, we have used the transgenic zebrafish line *Tg(nkx2.2a:mEGFP)* that expresses GFP in the nervous system (Ng et al. 2005) and revealed neurotoxicity (with reduced GFP expression in the spinal cord) caused by increasing the concentration of E2 (Fig. 17.2D–F), which was not apparent when visualized under normal light (Fig. 17.2A–C). Similarly, as shown in Figure 17.2G–H, the inhibitory effects of E2 on the vascularization and size of the liver would have been difficult to detect, or gone undetected, without the aid of fluorescence using

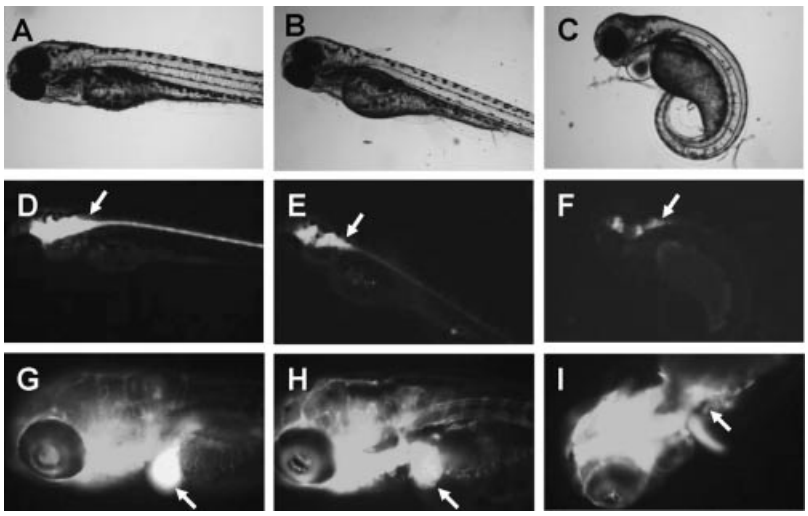


Figure 17.2. Use of fluorescent transgenic embryos/larvae for toxicological screen. (**A–F**) Estrogen-induced developmental toxicity in *Tg(nkx2.2a:mEGFP)* embryos. Transgenic embryos were treated with E2 at concentrations of 0 μg/L (ethanol vehicle) (**A**, **D**), 1.9 μg/L (**B**, **E**), and 2.7 μg/L (**C**, **F**) from 3 to 96 hpf (hour postfertilization). Panels **A**–**C** were captured under normal light while panels **E**–**H** were the same embryos/larvae captured under excitation light for GFP observations. Panels **D**–**F** show decreasing fluorescence and morphological disruption in the brain and spinal cord by increasing concentrations of E2. Arrows indicate the junction of brain and spinal cord. (**G**–**I**) Effect of E2 on vascularization and liver development in embryos from two-color double transgenic strain, *Tg(Fli:gfp; lfabp:rfp)*. Transgenic embryos were treated with E2 at concentrations of 0 μg/L (ethanol vehicle) (**G**), 1.4 μg/L (**H**), and 2.7 μg/L (**I**) from 3 to 96 hpf. Pictures were taken under conditions appropriate for GFP observation in the blood vessels and thus RFP expression in the liver is displayed in yellow (panel **G**) (arrows) under this condition. Decreasing fluorescence was observed in the liver with increasing concentration of E2, indicating affected vascularization and liver size. (For color detail, please see the color plate section.)

embryos of the two-color double transgenic zebrafish line *Tg(fli1:gfp; lfabp:rep)* (Lawson and Weinstein 2002; Korzh et al. 2008).

In published reports, transgenic zebrafish embryos expressing a GFP linked to the promoter of the aryl hydrocarbon receptor have been used to identify TCDD-targeted tissues (Mattingly et al. 2001). *Tg(neurog1:gfp)* and *Tg(shh:gfp)* zebrafish have also been used to investigate TCDD-induced neurotoxicity by assessing changes in *sonic hedgehog* (*shh*) and *neurogenin* expression in the zebrafish brain (Hill et al. 2003). Recently, *Tg(hsp70:gfp)* zebrafish have been reported to help identify cadmium-induced toxicity in sensory cells of the olfactory system and the lateral line following brief exposure to cadmium (Blechinger et al. 2007).

The application of these fluorescent transgenic fish for screening of specific tissue or organ toxicity will be most useful for understanding the toxicology of newly synthesized industrial and pharmaceutical compounds. The amenability of these transgenic embryos to automated high-throughput screening makes them highly desirable for drug discovery and toxicology (Love et al. 2004).

ADVANTAGES OF TRANSGENIC BIOMONITORING FISH

Limitations of conventional methods to water pollution detection spurred the concept of using biomonitoring fish. Presently, the major approach to monitor the quality of aquatic environments involves the testing of water, sediment, or tissue for residue levels using analytical chemical methods. However, the analytical chemistry approach is generally expensive and slow, and the process also requires acquisition of samples, transportation to the analytical facility, sample processing, data collection, and data analysis.

Wild fish caught in polluted water bodies are also used to evaluate the presence of pollutants. Biochemical assays, such as enzymatic assays involving ethyoxyresorufin-O-deethylase, superoxide dismutase, catalase tests, and glutathione peroxidase, are performed on tissues to quantify the activity of the enzymes induced by the various compounds (Kelly et al. 1998). Detection of biomarkers in tissues or the whole organism has also been used. For example, the levels of *choriogenin* mRNA and Vtg protein can be quantified in male livers and blood samples, respectively, to evaluate the level of estrogenic compounds (Kurauchi et al. 2005). Such biochemical or molecular assays require special equipment and training that are only available in established laboratories. Samples have to be handled with great care to prevent denaturation or proteolysis of the tissue. There are also limitations to the interpretation of these data because various factors such as individual variability, physiological, genetic, and metabolic factors have considerable effects on the results.

The advantages of using a biomonitoring transgenic fish system over conventional methods are apparent. Firstly, it does not require the sacrifice of the organisms and can allow for continuous monitoring of the same site with the same fish. Therefore, biological and technical variations are reduced, hence requiring fewer fish for validation and testing. In addition, the possibility of reusing the biomonitoring transgenic fish for repeated monitoring makes this approach cost-effective.

Secondly, in vivo testing reflects the bioavailability of the chemical and demonstrates the potency of the chemical better than in vitro assays. Since the physiology of the whole organism is taken into account, toxicokinetic mechanisms such as uptake, distribution, conversion, and metabolism of the pollutants can be studied as well. This is important in light of the fact that the toxicity of many compounds in vivo is influenced by biological processes such as biotransformation and bioaccumulation.

Thirdly, unlike analytical chemistry, which measures specific, selected, and characterized pollutants, and yields no biological or toxicological information, the use of a biomonitoring transgenic system does not require the pollutants to be characterized and it does yield such information. This is helpful since aquatic pollutants are usually found in a mixture of characterized and uncharacterized compounds in different abundances that vary in toxicity. A biomonitoring transgenic system can serve as a front-line alarm sentinel in the presence of compounds that are sufficient to exert biological impact. This will be used to justify further action and promote more detailed analyses by other means.

Fourthly, since the biomonitoring transgenic system works at the level of gene activation, it is considered to be a first line response to environmental

stressors before any adverse changes occur at the organismal and population level. From an ecological perspective, the use of a biomonitoring transgenic system can serve as an early and sensitive indicator to warn of potential problems and, subsequently, to prevent adverse impacts on biodiversity by early mitigation efforts.

Finally, setting up husbandry facilities for fish is relatively simple and inexpensive. The testing of water sources for transgenic fish does not need intricate equipment or require sophisticated training. Data analysis can be fast because of the directly observable fluorescent color change and without the need of sophisticated chemical and biological assays. The biggest advantage of the biomonitoring fish is the feasibility of developing an on-site and/or online monitoring system, thus detecting and immediately reporting aquatic contamination.

PERSPECTIVES

Designer Promoters in Transgenic Fish

Currently, most of the transgenic fish developed for environmental monitoring used native gene promoters that are inducible by certain chemicals. The critical components in the inducible promoters are sequence motifs, so-called response elements (REs), to react to certain environmental signals. For example, the *cyp1a1* promoters from many different species contain multiple xenobiotic response elements within 3 kb of upstream sequence (e.g., Zeruth and Pollenz 2005); this is expected as cytochrome P450 1a1 (CYP1a1) is an essential enzyme involved in xenobiotic metabolism and has been used as a primary indicator of environmental exposure to PAHs.

However, the capability of monitoring environmental pollution by the transgenic approach is limited by the availability of suitable promoters to drive reporter genes. To overcome this limitation, a designer promoter approach may be used to develop transgenic fish for monitoring a broader range of chemicals. The idea of designer promoters is to create artificial promoters by incorporation of different REs to respond to one or more category of pollutants. It is also of interest to create a better and stronger promoter by recruiting more copies of REs to enhance gene transcription and thus to improve the sensitivity of environmental monitoring.

There have already been attempts to create transgenic fish using artificial promoters. Carvan et al. (2000b) constructed several vectors containing artificial REs for aromatic hydrocarbon, electrophiles, or metals and tested their sensitivity to their respective chemicals in zebrafish cell culture; however, no established transgenic line was reported from this study. Leger et al. (2000) have reported on a stable line of transgenic zebrafish using an artificial promoter consisting of just three tandem repeats of EREs attached to a minimal promoter that was to test estrogenic activity. Recently, the EPREs used by Carvan et al. (2000b) have been linked to a Luciferase–GFP fusion protein gene to develop stable transgenic zebrafish in which expression can be induced by oxidative stress or mercury treatment (Kusik et al. 2008). All of these examples prove the feasibility of using artificial or designer promoters to develop transgenic biomonitoring fish. As understanding of gene regulatory systems progresses, it is likely that designer promoters will be developed to produce more suitable transgenic fish for environmental monitoring in the near future. The potentially useful REs for designer promoters are summarized in Table 17.2.

Multicolor Transgenic Biomonitoring Fish

Since the cloning of the first *GFP* cDNA from *Aqueorea victoria,* a series of either point or random mutations on the chromophore structure have been generated to alter or improve its fluorescent properties (Low and Gong 2005). Variants such as the blue fluorescent protein, cyan fluorescent protein, and yellow fluorescent protein have been created to exhibit different excitation and emission peaks for multicolor tagging experiments. Nature has also provided a red fluorescent protein (RFP, also commonly known as dsRed) from a species of sea anemone, *Discosoma sp*. (Matz et al. 1999). The structure of dsRed has also been altered to improve its stability as well as its signal intensity, and eventually these modifications led to an array of monomeric fluorescent proteins (the mFruit series) with emission peaks ranging from 537 nm to 610 nm (Shaner et al. 2004). Thus, the availability of these new fluorescent proteins provides more tools to develop fluorescent transgenic fish with different colors. In future, it is also possible to generate multicolors in the same transgenic fish

Table 17.2. Summary of Response Element Responses to Environmental Pollutants.

Response Elements (REs)	Consensus Sequences 5′–3′	Activating Agents
Estrogen response element (ERE)	GGTCANNNTGACC	Estrogenic compounds, chlorinated aromatic hydrocarbons, and insecticides
Metal response element (MRE)	TGCRCNC	Heavy metals
Xenobiotic response elements (XRE aka AHRE, DRE)	(T/G)NGCGTG	Polycyclic aromatic hydrocarbons, dioxins, and halogenated aromatic hydrocarbons
Electrophile response elements (EPRE aka ARE)	GTGACNNNGC	Planar aromatic hydrocarbons, potent electrophiles (heavy metals, quinones, diphenols)
Glucocorticoid response element (GRE)	GRACANNNTGTYC	Steroids, e.g., glucocorticoids androgen, mineralocorticoids, and progestins
Heat shock reponse element (HSE)	GAANNTTCNNGAA	External stress (e.g., high temperature)
Hypoxia response element (HRE)	RCGTG	Low oxygen level
Thyroid hormone response elements (TREs)	AGGTCANNNAGGTCA AGGTCATGACCT TGACCA($_{N4-6}$)AGGTCA	Thyroid—pharmaceutical
Peroxisome proliferator response element (PPRE)	AGGTCANAGGTCA	Peroxisome proliferator receptor ligands, e.g., prostaglandins and non-steroidal anti-inflammatory drugs(NSAID)
Retinoic acid response elements (RAREs)	RGGTCA($N_{0–8}$) RGGTCA	Retinoic acid and other retinoids—natural and pharmaceutical
Retinoid X response elements (RXRES)	GGGGTCAAAGGTCA GGGGTCATGGGGTCA	Retinoic acid and other retinoids—natural and pharmaceutical

Note: Within each consensus sequence: N = A,T,G, or C; R = A or G; Y = T or C.

to monitor different varieties of pollutants. This can be achieved either by coinjection of DNA constructs with two or more different fluorescent protein genes or by classic breeding of two transgenic lines with different colors. The feasibility of generating multicolor transgenic fish has been demonstrated in our laboratory. One transgenic zebrafish line has GFP expression in skin cells and RFP expression in muscle cells (Wan et al. 2002), and another one has GFP expression in the exocrine pancreas and RFP expression in the liver (Korzh et al. 2008). Recently, we have also generated two-color transgenic zebrafish by cross breeding two different transgenic lines to label insulin- and somatostatin-producing cells with different fluorescent proteins (Li et al. 2009). This research demonstrated the feasibility of developing multicolor transgenic fish to monitor multiple environmental pollutants simultaneously.

Microarrays and Toxicogenomics

Instead of studying the response of a single gene to an environmental toxicant in one experiment, DNA microarray technology is able to assess the responses of thousands of genes or the whole genome

simultaneously. Hence, a microarray experiment can provide a more comprehensive view of the global gene response to the toxicant. Since each class of toxicant has its own mode of action and toxicity and hence deregulates the expression of a specific set of genes, microarray experiments can capture the molecular "fingerprint" or "signature" consisting of the expression profiles of the deregulated genes responding to each class of toxicants. With the help of bioinformatics, this molecular "fingerprint" or "signature" can be used to decipher mechanisms of action and/or toxicity, infer health risk, identify biomarkers (highly sensitive and responsive genes), and predict or identify unknown compounds that may share a similar mode of action and toxicity. Gene expression-based toxicogenomics approach has been applied to many fish, such as carp (Moens et al. 2006), rainbow trout (Benninghoff and Williams 2008), Atlantic salmon (Mortensen and Arukwe 2008), European flounder (Falciani et al. 2008), fathead minnow (Mager et al. 2008), stickleback (Brown et al. 2008), medaka (León et al. 2008), and zebrafish (Lam et al. 2006, 2008; Yang et al. 2007; Wang et al. 2008).

Compared to classical survival–mortality endpoints in toxicological tests, the application of microarrays offers more insights into toxicity by providing modes of action and mechanistic information that will enable more robust and in-depth risk assessments. With gene information and homolog mapping, researchers could reduce much of the uncertainty, or at the least appreciate the limits better, when extrapolating from animal to human conditions or from laboratory to the actual field environment, a key problem in current risk assessment practices (Oberemm et al. 2005). On the basis of the microarray data, sets of biomarker genes that are highly responsive to specific stressors can be identified, and their expression profiles used as "standard reference" to detect the presence of certain toxicants or predict the mode of action of unknown compounds. Selected biomarkers associated with certain classes of compounds or key molecules involved in specific adaptive responses can be developed into rapid and sensitive PCR-based detection kits. Moreover, with the current whole genome sequencing projects and availability of cDNA sequences from a large number of fish species, it is possible to design "universal" primers with conserved sequences that are applicable to most, if not all, species of fish. Finally, gene promoters from new biomarker genes that respond to different stressors or toxicants can be characterized and used for developing new biomonitoring transgenic fish.

REFERENCES

Amanuma K, Takeda H, Amanuma H, and Aoki Y. 2000. Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. Nat Biotechnol. 18: 62–65.

Amsterdam A, Lin S, and Hopkins N. 1995. The *Aequorea victoria* green fluorescent protein can be used as reporter in live zebrafish embryos. Dev Biol. 171: 123–129.

Benninghoff AD and Williams DE. 2008. Identification of a transcriptional fingerprint of estrogen exposure in rainbow trout liver. Toxicol Sci. 101: 65–80.

Blechinger SR, Kusch RC, Haugo K, Matz C, Chivers DP, and Krone PH. 2007. Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. Toxicol Appl Pharmacol. 224: 72–80.

Blechinger SR, Warren JT, Kuwada JY, and Krone PH. 2002. Developmental toxicology of Cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Perspect. 110(10): 1041–1046.

Brown MM, Williams TD, Chipman JK, Katsiadaki I, Sanders M, and Craft JA. 2008. Construction of subtracted EST and normalised cDNA libraries from liver of chemical-exposed three-spined stickleback (*Gasterosteus aculeatus*) containing pollutant-responsive genes as a resource for transcriptome analysis. Mar Environ Res. 66: 127–130.

Carvan MJ III, Dalton TP, Stuart GW, and Nebert DW. 2000a. Transgenic zebrafish as sentinels for aquatic pollution. Ann NY Acad Sci. 919: 133–147.

Carvan MJ III, Solis WA, Gedamu L, and Nebert DW. 2000b. Activation of transcription factors in zebrafish cell cultures by environmental pollutants. Arch Biochem Biophys. 376(2): 320–327.

Dahm R and Geisler R. 2006. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol. 8: 329–345.

Falciani F, Diab AM, Sabine V, Williams TD, Ortega F, George SG, and Chipman JK. 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene

responses following exposure to model toxicants. Aquat Toxicol. 90: 92–101.

Gong Z, Ju B, and Wan H. 2001. Green fluorescent protein (GFP) transgenic fish and their applications. Genetica. 111: 213–225.

Gong Z, Wan H, Tay TL, Wang H, Chen M, and Tong Y. 2003. Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. Biochem Biophys Res Commun. 308(1): 58–63.

Gong Z, Wu YL, Mudumana SP, and Lin S. 2004. Transgenic fish for developmental biology studies. In: Fish Development and Genetics: The Zebrafish and Medaka Model (eds. Gong Z and Korzh V). World Scientific, Singapore. pp 476–516.

Hill A, Howard CV, Strahle U, and Cossins A. 2003. Neurodevelopmental defects in zebrafish (*Danio rerio*) at environmentally relevant dioxin (TCDD) concentrations. Toxicol Sci. 76: 392–399.

Ireland HE, Harding SJ, Bonwick GA, Jones M, Smith CJ, and Williams JHH. 2004. Evaluation of heat shock protein 70 as a biomarker of environmental stress in *Fucus serratus* and *Lemna minor*. Biomarkers. 9(2): 139–155.

Kelly SA, Havrilla CM, Brady TC, Abramo KH, and Levin ED. 1998. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Perspect. 106: 375.

Knight J. (2003) Glofish casts light on murky policing of transgenic animals. Nature. 426: 372.

Kobayashi D and Takeda H. 2008. Medaka genome project. Brief Funct Genomic Proteomic. 7(6): 415–426.

Korzh S, Pan X, Garcia-Lecea M, Winata CL, Pan X, Wohland T, Korzh V, and Gong Z. 2008. Requirement of vasculogenesis and blood circulation in late stages of liver growth in zebrafish. BMC Dev Biol. 8: 84.

Kurauchi K, Nakaguchi Y, Tsutsumi M, Hori H, Kurihara R, Hashimoto S, Ohnuma R, Yamamoto Y, Matsuoka S, Kawai S, Hirata T, and Kinoshita M. 2005. In vivo visual reporter system for detection of estrogen-like substances by transgenic medaka. Environ Sci Technol. 39: 2762–2768.

Kusik BW, Carvan MJ III, and Udvadia AJ. 2008. Detection of mercury in aquatic environments using EPRE reporter zebrafish. Mar Biotechnol. 10: 750–757.

La Porte PF. 2005. *Mytilus trossulus* hsp70 as a biomarker for arsenic exposure in the marine environment: laboratory and real-world results. Biomarkers. 10(6): 417–428.

Lam SH, Mathavan S, Tong Y, Li H, Karuturi RK, Wu Y, Vega VB, Liu ET, and Gong Z. 2008. Zebrafish whole-adult-organism chemogenomics for large-scale predictive and discovery chemical biology. PLoS Genet. 4(7): e1000121.

Lam SH, Winata CL, Tong Y, Korzh S, Lim WS, Korzh V, Spitsbergen J, Mathavan S, Miller LD, Liu ET, and Gong Z. 2006. Transcriptome kinetics of arsenic-induced adaptive response in zebrafish liver. Physiol Genomics. 27: 351–361.

Lawson ND and Weinstein BM. 2002. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 248: 307–318.

Legler J, Broekhof JLM, Brouwer A, Lanser PH, Murk AJ, van der Saag PT, Vethaak AD, Wester P, Zivkovic D, and van der Burg B. 2000. A novel in vivo bioassay for (xeno-)estrogens using transgenic zebrafish. Environ Sci Technol. 34: 4439–4444.

León A, Wu PS, Hall LC, Johnson ML, and Teh SJ. 2008. Global gene expression profiling of androgen disruption in Qurt strain medaka. Environ Sci Technol. 42: 962–969.

Li Z, Wen C, Peng J, Korzh V, and Gong Z. 2009. Generation of living color transgenic zebrafish to trace somatostatin-expressing cells and endocrine pancreas organization. Differentiation. 77: 128–134.

Love DR, Pichler FB, Dodd A, Copp BR, and Greenwood DR. 2004. Technology for high-throughput screens: the present and future using zebrafish. Curr Opin Biotechnol. 15: 564–571.

Low BC and Gong Z. 2005. Reporter gene system: Green fluorescent protein. In: Encyclopedia of Molecular Cell Biology and Molecular Medicine (ed. Meyers RA). Vol 12. Wiley-VCH, Weinheim, Germany. pp 215–247.

Mager EM, Wintz H, Vulpe CD, Brix KV, and Grosell M. 2008. Toxicogenomics of water chemistry influence on chronic lead exposure to the fathead minnow (*Pimephales promelas*). Aquat Toxicol. 87: 200–209.

Mattingly CJ, McLachlan JA, and Toscano WA Jr. 2001. Green fluorescent protein (GFP) as a marker of aryl hydrocarbon receptor(AhR) function in developing zebrafish (*Danio rerio*). Environ Health Perspect. 109(8): 845–849.

Matozzo V, Gagné F, Marin MG, Ricciardi F, and Blaise C. 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ Int. 34: 531–545.

Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, and Lukyanov SA.

1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol. 17: 969–973.

Moens LN, van der Ven K, van Remortel P, Del-Favero J, and De Coen WM. 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. Toxicol Sci. 93: 298–310.

Mortensen AS and Arukwe A. 2007. Targeted salmon gene array (SalArray): a toxicogenomic tool for gene expression profiling of interactions between estrogen and aryl hydrocarbon receptor signalling pathways. Chem Res Toxicol. 20: 474–488.

Nam YK, Maclean N, Fu C, Pandian TJ, and Equia MRR. 2007. Development of transgenic fish: scientific background. In: Environmental Risk Assessment of Genetically Modified Organisms: Vol. 3, Methodologies for Transgenic Fish (eds. Kapuscinski AR, Hayes KR, Li S, and Dana G). Biddles Press, King's lynn, UK. pp 61–94.

Ng ANY, de Jong-Curtain TA, Mawdsley DJ, White SJ, Shin J, Appel B, Dong PDS, Stainier DYR, and Heath JK. 2005. Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. Dev Biol. 286: 114–135.

Oberemm A, Onyon L, and Gundert-Remy U. 2005. How can toxicogenomics inform risk assessment? Toxicol Appl Pharmacol. 207: 592–598.

Shaner NC, Cambell RE, Steinbach PA, Giepmans BNG, Palmer AE, and Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nature Biotechnol. 22: 1567–1572.

Salam MA, Sawada T, Ohya T, Ninomiya K, and Hayashi S. 2008. Detection of environmental estrogenicity using transgenic medaka hatchlings (*Oryzias latipes*) expressing the GFP-tagged choriogenin L Gene. J Environ Sci Health A Tox Hazard Subst Environ Eng. 43: 272–277.

Sanger. 2009. http://www.sanger.ac.uk/Projects/D_rerio/.

Seok SH, Baek MW, Lee HY, Kim DJ, Na YR, Noh KJ, Park SH, Lee HK, Lee BH, Ryu DY, and Park JH. 2007. Quantitative GFP fluorescence as an indicator of arsenite developmental toxicity in mosaic heat shock protein 70 transgenic zebrafish. Toxicol Appl Pharmacol. 225: 154–161.

Seok SH, Baek MW, Lee HY, Kim DJ, Na YR, Noh KJ, Park SH, Lee HK, Lee BH, and Park JH. 2008. In vivo alternative testing with zebrafish in ecotoxicology. J Vet Sci. 9: 351–357.

Seok SH, Park JH, Baek MW, Lee HY, Kim DJ, Uhm HM, Hong JJ, Na YR, Jin BH, Ryu DY, and Park JH. 2006. Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebrafish. J Biotechnol. 126: 406–413.

Thompson S, Tilton F, Schlenk D, and Benson WH. 2000. Comparative vitellogenic responses in three teleost species: extrapolation to in situ field studies. Mar Environ Res. 51: 185–189.

Tong Y, Shan T, Poh YK, Yan T, Wang H, Lam SH, and Gong Z. 2004. Molecular cloning of zebrafish and medaka vitellogenin genes and comparison of their expression in response to 17β-estradiol. Gene. 328: 25–36.

Udvadia AJ and Linney E. 2003. Windows into development: historic, current, and future perspectives on transgenic zebrafish. Dev Biol. 256: 1–17.

Ueno T, Yasumasu S, Hayashi S, and Iuchi I. 2004. Identification of choriogenin *cis*-regulatory elements and production of estrogen-inducible, liver-specific transgenic medaka. Mechan Dev. 121: 803–815.

UTGB Medaka. 2008. http://utgenome.org/medaka/

Wakamatsu Y, Pristyazhnyuk S, Kinoshita M, Tanaka M, and Ozato K. 2001. The see-through medaka: a fish model that is transparent throughout life. Proc Natl Acad Sci USA. 98(18): 10046–10050.

Wan H, He J, Ju B, Yan T, Lam TJ, and Gong Z. 2002. Generation of two-color transgenic zebrafish using the green and red fluorescent protein reporter genes gfp and rfp. Mar Biotechnol. 4(2): 146–154.

Wang RL, Bencic D, Biales A, Lattier D, Kostich M, Villeneuve D, Ankley GT, Lazorchak J, and Toth G. 2008. DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. Environ Toxicol Chem. 27: 664–675.

Weber R, Gaus C, Tysklind M, Johnston P, Forter M, Hollert H, Heiniseh E, Holoubek I, Lloyd-Smith M, Masunaga S, Moccarelli P, Santillo D, Seike N, Symons R, Torres JPM, Verta M, Varbelow G, Vijgen J, Watson A, Costner P, Woelz J, Wyeisk P, and Zennegg M. 2008. Dioxin- and POP-contaminated sites—contemporary and future relevance and challenges. Environ Sci Pollut Res. 15: 363–393.

Winn RN, Norris MB, Brayer KJ, Torres C, and Muller SL. 2000. Detection of mutations in transgenic fish carrying a bacteriophage λcII transgene target. Proc Natl Acad Sci USA. 97(23): 12655–12660.

Winn RN, Norris M, Muller S, Torres C, and Brayer K. 2001. Bacteriophage λ and plasmid pUR288 transgenic fish models for detecting *in vivo* mutations. Mar Biotechnol. 3: S185–S195.

Wittbrodt J, Shima A, and Schart M. 2002. Medaka-A model organism from the Far East. Nat Rev Genet. 3: 53–64.

Wu YL, Pan X, Mudumana SP, Wang H, Kee PW, and Gong Z. 2008. Development of a heat shock inducible gfp transgenic zebrafish line by using the zebrafish hsp27 promoter. Gene. 408: 85–94.

Yang L, Kemadjou JR, Zinsmeister C, Bauer M, Legradi J, Müller F, Pankratz M, Jäkel J, and Strähle U. 2007. Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. Genome Biol. 8: R227.

Zbikowska HM. 2003. Fish can be first-advances in fish transgenesis for commercial applications. Transgenic Res. 12: 379–389.

Zeng Z, Shan T, Tong Y, Lam SH, and Gong Z. 2005. Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. Environ Sci Technol. 39: 9001–9008.

Zeruth G and Pollenz RS. 2005. Isolation and characterization of dioxin-inducible CYP1a1 promoter/enhancer region from zebrafish (*Danio rerio*). Zebrafish. 2(3): 197–209.

Chapter 18

Transgenic Tilapia for Xenotransplantation

James R. Wright Jr., Olga Hrytsenko, and Bill Pohajdak

INTRODUCTION

In type I or insulin-dependent diabetes mellitus, the patient's insulin-producing β-cells within their pancreatic islets of Langerhans have been destroyed by an autoimmune process. This often occurs at a young age, and hence the disease is sometimes called juvenile-onset diabetes. The loss of β-cells results in low levels of insulin and high levels of blood glucose (hyperglycemia). Since the discovery of insulin, these patients are treated with multiple, daily insulin injections, which prevent the immediate life-threatening symptoms of the disease; however, over a period of many years, most patients develop secondary complications that are due to moment to moment variations in blood glucose levels. A better treatment for diabetes would be to replace the islets, which also function as blood glucose sensors responding to hyperglycemia by secreting more insulin and hypoglycemia by secreting less. One mechanism of replacing islets is to transplant whole pancreases. However, this procedure has some undesirable aspects that could theoretically be avoided by transplanting only the islets. For >30 years, islet transplantation has been the Holy Grail in diabetes management. Unfortunately, it is difficult to separate the islets from the remainder of the pancreas (islets represent only 1% and are scattered throughout). Current methods to "isolate" islets result in highly variable islet yields and quality. It has also proven difficult to prevent the islet grafts from rejecting; however, the advent of the Edmonton Protocol has greatly improved short-term outcomes and given hope to millions of type 1 diabetics (Shapiro et al. 2000); unfortunately, favorable long-term results are less consistent (Ryan et al. 2005).

One of the most pressing problems is an inadequate supply of islets, as shortages of human cadaveric donor pancreases will prevent widespread application. Currently, there are <5000 suitable donor pancreases available annually in North America and most experts believe that the supply cannot be dramatically increased. Each islet transplant usually requires islets from 2–3 donors and, since there are currently several million type I diabetic patients in North America (with >30,000 new patients added each year), allotransplantation of human islets cannot meet the clinical need. Many experts believe that a practical, inexpensive source of animal islets should be identified for clinical islet xenotransplantation (Rayat and Gill 2003). Nonhuman primates are unsuitable donors for ethical, logistical, and safety reasons. The pig could be an ideal donor, but

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

isolation of adult porcine islets is difficult and costly (Ricordi et al. 1990). Furthermore, government regulatory agencies will not allow slaughterhouse pigs to be donors, and donors will have to be produced indoors under specific pathogen-free (SPF) conditions. The cost will be astronomical, especially because of the relatively low yield per donor (Wright and Pohajdak 2001; Wright et al. 2004). Finally, there are concerns related to possible transmission of xenozoonotic diseases (Sihvonen 2004).

In the early 1990s, our laboratory developed a model for experimental islet xenotransplantation using tilapia as donors (Wright 1992). Islets from teleost fish like tilapia occur as multiple anatomically discrete organs called Brockmann bodies (BBs). Unlike harvesting mammalian islets, where it is necessary to perform problematic "isolation" procedures, BBs can be harvested directly by microdissection (Wright 1994) or mass-harvested from multiple donor fish simultaneously using a fast, inexpensive, and simple enzymatic method (Yang and Wright 1995). When tilapia BB fragments are transplanted under the kidney capsules of diabetic nude mice, we and others achieve uniform long-term normoglycemia with recipients demonstrating mammalian-like glucose tolerance profiles (Wright et al. 1992; Morsiani et al. 1995; Yang et al. 1997a,b). When transplanted into euthymic mice, this is an excellent model to study islet xenograft rejection and methods to abrogate rejection (Wright et al. 2004).

Much of our xenotransplantation research has focused on microencapsulation (i.e., enclosing the grafts prior to transplantation within semipermeable membranes with very small "pores" allowing diffusion of glucose, insulin, and oxygen, but preventing entry of larger molecules such as immunoglobulins and cells of the immune system). Using both microencapsulation and low-dose immunosuppression, we have achieved long-term graft function of encapsulated fish islets in diabetic rodents (Yang et al. 1997a, b; Yang and Wright 2002). In collaboration with Joe Leventhal at Northwestern University, we have also generated promising preliminary results transplanting encapsulated tilapia islets into diabetic cynomologus monkeys (Leventhal et al. 2006). One of the major problems encountered with encapsulation is that islets cannot revascularize after transplantation within encapsulation devices and are very susceptible to hypoxia. Because tilapia normally live in a very hypoxic environment (i.e., warm, stagnant water), tilapia islets tolerate hypoxia many fold better than mammalian islets and, thus, are better suited for encapsulation (Wright et al. 1998).

Although fish donors may seem unsuitable for clinical islet xenotransplantation, we believe that tilapia are the ideal future clinical donor source for several reasons. First is the projected cost of producing SPF donor pigs and of isolating their islets. As explained in a recent review (Wright et al. 2004), we conservatively project the cost of using fish donors to be about 100-fold less per clinical transplant. Secondly, from the perspective of transmitting xenozoonotic diseases, tilapia islets are likely safer than mammalian islets as the probability of transmitting disease decreases with increasing phylogenetic disparity. However, there is a major problem. Fish insulins are structurally dissimilar to human insulin and, although functional in man (Wright 2002a), might prove immunogenic. Specifically, tilapia and human insulins differ by 9 amino acids in the A-chain and 8 amino acids in the B-chain (Nguyen et al. 1995).

HISTORY OF THE TG FISH PROJECT (FUNDING AND PATENT ISSUES)

Because tilapia islets function in a manner surprisingly similar to mammalian islets and because of the marked cost advantage, we initially discussed the possibility of producing transgenic (TG) tilapia expressing a human insulin gene in 1994. Based upon early discussions, we decided that the more logical approach would be to clone, sequence, and "humanize" the tilapia insulin gene and then produce TG fish by microinjecting the humanized gene into fertilized eggs at the single-cell stage. A small start up grant (∼$10K) was obtained in 1994 from the IWK Children's Hospital in Halifax. This funding was used to clone and sequence the tilapia insulin gene. There was no initial attempt to publish the work as we wanted to patent the concept; however, this work was published once patent applications had been filed (Mansour et al. 1998).

In 1994, we came very close to obtaining global project funding from the London Life Insurance Company, which holds an annual cross-Canada research competition to fund a single new project. We

made a short list of two, but at the time of the site visit, our laboratory was in the process of moving and all we could show the site visit team was shelled in space where the future laboratory would be located in about a year. We were not funded.

In 1995, we were able to obtain two years of grant funding from the Juvenile Diabetes Foundation International (JDFI) in NYC to pursue the idea of producing the TG fish; the funding was $50K/year. Broad patent applications were filed by Dalhousie University and its partner institution, the IWK Health Centre. At about the same time, we were negotiating with Sandoz USA and Sandoz Canada to obtain pharmaceutical funding to support moving the project forward at a faster pace than could be done with a single small operating grant. Jean-Paul Castaigne, Vice President Medical and Regulatory Affairs for Sandoz Canada, championed the project and Sandoz Canada provided several small grants totaling $78K to keep the project afloat while we attempted to obtain major funding from the parent company in Basel, Switzerland. However, Sandoz was in the process of merging with another pharmaceutical giant, Ciba-Geigy, and was unable to make a final commitment for major funding until the merger was complete. Furthermore, Sandoz had expressed some concerns about the strength and breadth of our patent application; therefore, even though Sandoz Canada had assured us that they could support the work while the decision was being made, the patent issues caused us grave concern about waiting for up to a year for a final decision.

While all of this was happening, a small biotechnology company, VivoRx in Santa Monica, California, became acutely interested in our technology. VivoRx's main product in development was a microencapsulation device designed to provide immunoprotection for islet grafts. A major impediment to their success was the lack of an economical source of islets, which we believed could be resolved using TG tilapia islets. Because VivoRx had cash available immediately, Dalhousie's Technology Transfer Office signed a binding letter of intent with VivoRx to form a partnership and major funding was assured. However, our celebration was very short-lived, as once the letter was signed, VivoRx and the University could not agree on some licensing issues and the funding was not forthcoming. Furthermore, because of our contract for global funding from VivoRx, we were ineligible to apply to renew our grant from the JDFI. Eventually, the University and VivoRx came to terms and we expected the money to flow immediately. However, by this time, VivoRx was on the verge of imploding because of internal and external lawsuits, focused around allegations that the President had diverted funding from Mylan Laboratories' $20,000,000 investment in VivoRx's microencapsulation technology into his other company American Bioscience and was allegedly using this to pursue cancer research (Simon 1996; Armstrong 2003). VivoRx defaulted on their agreement to invest in our technology but eventually negotiated a small severance agreement (∼$100K) precluding us from suing them. This was used to pay off debts generated while the project was unfunded and then to provide minimal funding for the project going forward. Since we were legally bound to VivoRx and had burned our bridges with Sandoz/Novartis, we had to all but shut down our operations and seek new sources of funding. The IP rights eventually reverted to the Technology Transfer Office nine months after the default was official. Essentially, the years 1997–2000 were lost, but the project was maintained on "life support" with collateral funding for other islet transplantation research. We spent most of 1999 writing grants and the project was fully resuscitated in the year 2000, when we received funding from AquaNet, a Canadian Networks of Centers of Excellence grant to promote aquaculture in Canada (∼$100K/year × 3 years), and from a Collaborative Health Research Project grant, a new partnership between the Natural Sciences and Engineering Research Council (NSERC) and the Canadian Institutes for Health Research (CIHR; ∼$112K/year × 3 years). Dr. Wright also received new grants from CIHR and NSERC for other projects related to fish islet xenotransplantation. After this windfall, funding for xenotransplantation research became harder to obtain because of mounting concerns about potential health risks.

In the late 1990s, governments around the world were deciding how to regulate xenotransplantation, as there were theoretical infectious disease risks (Wright 2002b, 2004). In 1997, a paper had been published, showing that porcine endogenous retrovirus (PERV) could be transmitted to human cells cocultured with pig cells (Patience et al. 1997). Although not specifically relevant to fish-to-human xenotransplantation, the paper was a bombshell to

the field of xenotransplantation, as there was speculation that pig-to-human xenotransplantation could start a worldwide AIDS-like epidemic; this halted xenotransplantation clinical trials in the developed world and the focus of xenotransplantation research changed to studying PERV. Because of the uncertainty, the pharmaceutical industry lost interest and ceased to be major funders. A decade of research on PERV suggests that it is not likely a significant issue (Fishman and Patience 2004), but there is still uncertainty (Wilson 2008).

With the funding obtained in 2000 and subsequent renewal of several of these grants, we were able to produce TG tilapia expressing a humanized insulin gene (Pohajdak et al. 2004) and begin an intermittent breeding program (see below). Our first US patent (Wright and Pohajdak 2000) was issued on January 18, 2000, which provided patent coverage for the method for harvesting fish islets, and the subsequent US patent, covering the actual TG tilapia (Wright and Pohajdak 2002), was issued on November 5, 2002. No real attempt was made to patent the fish in Canada as patenting life forms is not legal.

In 2004, Dr. Wright began negotiations to move to the University of Calgary and the Calgary Health Region as the Head of the Department of Pathology and Laboratory Medicine for both entities, with the move occurring in June 2005. In early 2005, the Wright laboratory shut down to negotiate equipment ownership with the Hospital and the University. All work related to the TG fish project was centralized to the Pohajdak laboratory but there was no specific funding to move the project forward. As we downsized our non-TG tilapia hatchery in Halifax, we provided Dr. Joe Leventhal, a transplant surgeon at Northwestern University in Chicago, encapsulated tilapia islets for a pilot study, transplanting them into diabetic cynomologous monkeys (Leventhal et al. 2006).

The move slowed the research as facilities and personnel needed to be duplicated in two cities. Because of the "energy economic boom" and massive project cost inflation in Calgary at that time, the "fit out" of the new building where the primary tilapia housing facility was to have been located was not completed. The recirculation system for housing tilapia in Calgary (designed by Northern Tilapia (Bond Head, ON), built and tested in a warehouse in Ontario, disassembled, and then shipped to Calgary in 2006) is still in storage. The TG tilapia colony remains in Halifax, but finding funding to maintain a breeding program has been problematic. Granting agencies that fund basic research are uninterested since it is not a hypothesis-based research. Our prior experience with corporate partners has made us wary of these relationships, and the short period of time left on our patent (i.e., since its "priority date"), the long period of time required to characterize and optimize our TG fish, and the uncertainties surrounding the whole field of xenotransplantation make our work unattractive to "for profit" entities. Therefore, the logical partners for our work are "non profit" entities interested in novel possibilities for curing diabetes.

In early 2006, the Juvenile Diabetes Research Foundation (JDRF) in New York City sent out a call for letters of intent to fund a research network for islet xenotransplantation studies in lower primates. We submitted a letter of intent proposing a research network building upon our preliminary primate studies. We proposed that the TG tilapia facility in Halifax would function as a core facility for TG tilapia islets (once the breeding program was completed) and that the islet transplantation laboratory in Calgary would function as a core facility for harvesting and shipping non-TG tilapia islets to network investigators around North America. We were not invited to submit a network grant, as the JDRF told us that the "cell biology" of how wild-type tilapia islets function was poorly understood and that it was premature to do primate studies. However, the JDRF asked us to rapidly (<4 weeks) prepare an operating grant proposal to address cell biology. We were funded and told that the JDRF would consider funding our breeding program once we had a better understanding of how tilapia islets maintain normoglycemia. We were fortunate that JDRF allowed us to use carryover funds to do one round of breeding, as many of the TG fish in our colony were old and losing their fecundity.

PRODUCTION OF TG TILAPIA

Our approach was to produce TG tilapia with a "humanized" tilapia insulin gene rather than inserting a human insulin gene. We were not aware of this approach having ever previously been used to make TG fish, but it seemed logical for our needs. Tilapia

and human insulins differ by 9 amino acids in the A-chain and 8 amino acids in the B-chain. Therefore, Dr. Pohajdak's laboratory cloned and sequenced the tilapia insulin gene and modified it by site-directed mutagenesis so that it codes for "humanized" insulin. Using tilapia preference codons, we modified only the codons for discrepant amino acids; the remainder of the gene, including its regulatory sequences, was unaltered. All vertebrate insulin genes code for a proinsulin where the B and A chain are separated by a C chain. We did not alter the prepro-insulin leaders nor the C-chain so that our construct maintains similar recognitions sites for the endopeptidases; this was done so that our TG fish would process humanized insulin as their own native tilapia insulin; however, this approach would result in the product being [desThrB30] human insulin (i.e., a human insulin molecule missing the final amino acid on the B-chain). However, since human and porcine insulin activities are essentially equal in humans and since porcine insulin's amino acid sequence also differs at the 30th amino acid on the B-chain, this was not a concern. The other reasons for taking this approach was that we wanted to, after removing the islet tissue for transplantation, be able to potentially use the rest of each donor productively (e.g., sell the edible part of the fish for animal or human consumption and use the inedible parts to make fertilizer) to further subsidize the cost of islet production. We knew that if the fish contained any human DNA, it could never enter the food chain and we would need to burn the remainder of each donor, resulting in additional costs rather than cost savings.

While Dr. Pohajdak's laboratory was creating the transgene, Dr. Wright visited Dr. Garth Fletcher's laboratory to learn gene microinjection technique. Dr. Fletcher and his associate Dr. Margaret Shears demonstrated the method they used to microinject salmon eggs via the micropyle (Shears et al. 1992), and we modified this slightly to accommodate the smaller size of tilapia eggs. The conditions used to perform microinjections mimicked those of Rahman and MacLean (1992). The animal husbandry methods used to generate fertilized eggs, our methods for transgene microinjection, and how we handled the eggs after microinjection have been previously described in detail (Pohajdak et al. 2004).

One key to success was modifying our screening method. Initially, all eggs from a single mating and microinjection session were housed together, grown to ∼3–4 months of age, fin clipped, and then the clippings were screened. However, after a year of performing microinjections, we had filled our aquatic facility with tanks of juvenile tilapia and had no TG fish to show for our efforts. We knew that producing TG fish was not efficient (i.e., a few percent of microinjected eggs) but this seemed impossible. We noted that the Fletcher laboratory screened for "positives" primarily by phenotype (i.e., large size), that we did not expect our TG tilapia would have any phenotypic changes, and that the group housing method we were using resulted in very high mortality rates with often a few large aggressive fish decimating their "tank mates" before they were large enough to safely fin clip and screen. At this point, we found an article describing a whole body enzymatic digestion method for screening zebrafish larvae (Kawakami and Hopkins 1996), and using our knowledge of tilapia larval development (Morrison et al. 2001), we were able to modify the method to screen larval tilapia and immediately achieved appropriate rates of transgenesis.

Dalhousie University, located by the Atlantic Ocean and having a large Oceanography department, has an excellent aquatic animal facility (the Aquatron); however, it is mostly a salt water facility and our operation was its major freshwater user. Freshwater for our tilapia facility was achieved by dechlorinating city water, heating it to 28°C, and then pumping it to our tanks as a flow-thorough system. By October 2000, we had produced ∼15 potential founders. We housed each of these valuable TG fish separately to maximize growth and prevent any possibility of being harmed by aggressive tank mates. This resulted in each fish being in a pristine 20 gallon tank hooked in series to the Aquatron-heated freshwater system. Unfortunately, on Saturday October 7, 2000, the city of Halifax had a water main break, affecting its main water supply at Kearny Lake reservoir. They immediately reestablished water flow from a backup reservoir at Chain Lakes but the water had a brown discoloration and the city's staff made a decision to markedly increase the chlorine levels without warning. This overwhelmed the Aquatron's decholorinators and the tilapia facility

was quickly circulating chlorinated water, killing half of the TG fish. Ironically, less valuable, wild-type tilapia, housed at extremely high densities, were largely unaffected as the chlorine bound to organic particulate matter in the water. Only fish housed at low densities were affected.

One surviving founder, NT 56, could pass the transgene to offspring. Many of his F1 offspring (i.e., from multiple matings with different wild-type females) produced physiologic serum levels of human insulin as measured by a human-specific RIA. Immunoperoxidase staining for human insulin using several antibodies that do not cross-react with tilapia insulin showed that human insulin production was specific to the β-cells in the BBs (Pohajdak et al. 2004). However, as expected, the BBs simultaneously produced both humanized and tilapia insulin and, thus, would be suboptimal for clinical transplantation. Our eventual goal is to produce TG tilapia with BBs that secrete only human insulin. To achieve this, we will need to either knockout the native tilapia insulin gene or permanently silence it (see below). Based upon what we know about glucose homeostasis in tilapia (Alexander et al. 2006) and the ability of mammalian insulin to maintain normal metabolism and growth in fish after surgical removal of all BBs (Kelly 1993), production of only human insulin is not expected to have any deleterious effect.

STATUS OF THE BREEDING PROGRAM

Our overall objective is to develop lines of donor TG tilapia with these features: (i) useful, stable expression of the transgene, (ii) good survival and reproduction, (iii) uniform expression by individuals within a line, and (iv) a well-documented breeding program with a suite of genetic markers. Since we do not have expertise in tilapia genetics, we sporadically hired a consultant, Dr. Roger Doyle. Breeding requires significant funding, a large aquarium facility, and rapid screening capabilities. The combination of all three in a sustained fashion has been elusive.

We began by breeding NT56, a male, to as many wild-type females as possible to produce a population containing 50% TG heterozygous F1 offspring. These heterozygous fish were either mated or to be mated among themselves to produce 25% homozygous TG fish in the F2 generation. Screening needed to be done at two levels, the gene and the protein, to select breeders as not all "positive" fish produced good serum levels of human insulin. We initially had 12 F1 families and 4 F2 families. However, we had two major set backs. First, we lost three F1 families on September 4, 2003, due to a freshwater supply interruption. The second set back, on July 16, 2004, was catastrophic. About 10 days earlier, we were told that there was going to be a prolonged period when the Aquatron would have extreme limitations on its freshwater service and the water temperature would be dropped to increase dissolved oxygen levels and circulation would be minimal (a critical part of the freshwater system was failing and needed to be removed, sent off, rebuilt, and then reinstalled into the system; the down-time was unknown but would be substantial). We unwisely decided to very aggressively screen a large number of NT56 F1 and F2 offspring, all of which were known to carry the transgene, that were now large enough to breed to determine which ones had optimal levels of circulating human insulin for breeding. The insulin assay required 1 mL of blood, a significant volume for the fish, but we had not previously had mortalities. Whole families of valuable fish were bled with no mortalities about a week before the scheduled shutdown. Although phlebotomy is a stressful procedure, we believed that the week-long recovery time would be more than sufficient. However, as soon as the shutdown occurred, essentially all died within 24 hours.

We began breeding NT56 again, to create new F1 offspring and he sired more clutches; however, the old guy got so excited one night that he jumped out of his tank and was found dead on the floor on April 13, 2005. Interestingly, NT56, although he could pass the transgene to offspring, never possessed measurable human insulin in his circulation and was a mosaic; at necropsy, when histologic sections of his islet tissue were immunostained with noncross-reactive antibodies for either tilapia insulin or human insulin, most of the β-cells stained only for tilapia insulin but there was also a small population of very abnormal appearing β-cells that stained for human insulin (Wright et al. 2008).

Currently, we have 298 TG NT56 offspring from F1 and F2 generations. Approximately, 100 TG fish

potentially contain two alleles of the trangene in their genome (homozygotic) and therefore can be used to prepare a stable line of TG fish. To distinguish heterozygotic and homozygotic fish, we have recently developed a quantitative PCR method that allows us to estimate ratios, either humanized insulin gene: tilapia insulin gene and/or total insulin genes (humanized and native tilapia): reference gene in the tilapia genomic DNA.

FUTURE IMPROVEMENTS TO TG TILAPIA

Over the last few decades, the functions of novel proteins have been determined by experimentally disrupting the expression of the targeted genes (Muller 1999). Most of these "knockouts" (KOs) have been performed in mice, but recently this technology has been used in several other species. One of the major drawbacks for this technology is the need for embryonic stem (ES) cells, which are not easy to produce. Gene disruption in these ES cells usually involves using large genomic DNA that contains the mutation/deletion. Following transfection, cells are selected that have undergone homologous recombination. Since homologous recombination is a rare event (as compared to just gene insertion), this is labor-intensive. However, by using positive and negative selection methods, it is possible to efficiently screen for these rare events. Next, the ES cells are transplanted into developing blastulas and allowed to develop. The heterozygous offspring are screened and brother/sister mated to obtain homozygous KOs. This process is labor-intensive and slow.

KO technology is likely applicable to our goal but has proven difficult in fish. The culture conditions under which ES-cells have been established are species-dependent and unpredictable; in most instances, modification of a single variable has proven critical to eventual success. With initial funding from AquaNet, we made progress toward this goal (MacNeil 2002). As a starting point, Dr. Wright visited a top zebrafish laboratory (Paul Collodi, Purdue University) to learn ES-cell methodology and to gain "hands on" zebrafish experience. Unfortunately, we quickly found that the zebrafish technique was not applicable to tilapia without major modification at almost every step. For instance, the chorion surrounding the blastula is much thicker and is relatively resistant to digestion with pronase, their method of freeing the blastulas. Therefore, we completed a detailed study of the chorion and developed methods of dechorionating tilapia embryos (Morrison et al. 2003). In zebrafish, the blastulas are harvested at 3 hours after fertilization (roughly the 1000-cell stage); tilapia are not even approaching a blastula stage at 3 hours. We studied embryonic development in tilapia (Morrison et al. 2001) and found that the 1000 cell stage occurs at roughly 10 hours postfertilization in tilapia. Using some of the tissue culture conditions used to produce zebrafish and medaka ES-like cells, we were able to maintain undifferentiated tilapia blastula cells for up to 1 week (MacNeil 2002). Once putative tilapia ES-cells have been established, ES-cell markers including endogenous alkaline phosphatase activity (i.e., a marker expressed in ES-cells of all reported species), chromosomal euploidy, etc. can be used to confirm the nature of the cells. Formation of embryoid bodies (three-dimensional clusters formed when ES-cells are grown in suspension culture) can be used to establish pluripotency in vitro and teratoma (tumors composed of all three germ layers) formation after transplantation into athymic nude mice can be used to establish pluripotency in vivo. The second step, knocking out the native tilapia insulin gene in vitro, should be straightforward and we anticipate the mouse methodology will work, as this approach has been used for gene targeting in medaka ES-cells (Hong et al. 2004). The third step is to make germ-line chimeras. Based upon work funded by AquaNet, we do not believe that this will be difficult. Our initial plan had been to make chimeras using blastulas from red-pigmented and grey-pigmented tilapia, and we imported a pure red strain of tilapia for this purpose; however, a nightmarish level of government bureaucracy designed for imported salmonids effectively prevented us from using them during the 3-year term of the grant (Wright 2006). As a result, we used TRITC-labeled gray tilapia blastula cells (produced by microinjecting fertilized eggs at the single cell stage with TRITC-dextran), microinjected these into developing grey tilapia blastulas (i.e., 12 hours postfertilization), and then examined the resulting larvae (i.e., day 6 postfertilization) for the presence and distribution of labeled cells (Fig. 18.1). Because this method utilized a cytoplasmic label that diluted with each cell division, we

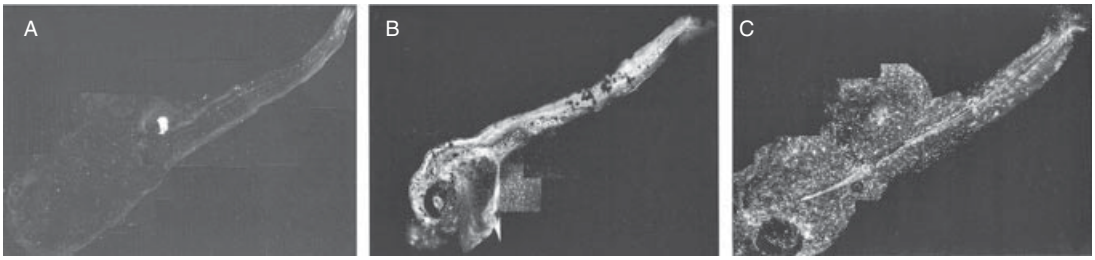


Figure 18.1. Confocal images showing fluorescence in a typical (**A**) negative control hatchling developing from a noninjected zygote, (**B**) positive control hatchling developing from a TRITC-injected zygote, and (**C**) chimeric hatchling developing from a noninjected zygote, which, at blastula stage, was microinjected with dispersed, TRITC-labeled cells from a positive control blastula (note the very wide distribution of labeled cells suggesting a high probability of germ cell involvement; MacNeil 2002).

could not test adult tilapia for germ-line expression; however, the very high degree of somatic expression strongly suggests germ cell expression. Once we have completed all three steps, we can combine these to KO the native tilapia insulin gene in our TG fish. Although we believe this approach will work, developing pluripotent tilapia ES cells would be time-consuming.

Recently, a new and improved method for generating gene knockdowns has been developed, often referred to as RNA interference (RNAi), involving a small interfering RNA (siRNA) (McManus and Sharp 2002). Although complex and involving several steps, the mechanism is based on the fact that small duplex RNA is recognized and removed by the cell's molecular machinery. The introduction of small (21–23 bases) antisense RNA complementary to the gene targeted causes the destruction of that mRNA. The complex is first recognized by the enzyme Dicer, which cleaves the mRNA. This small duplex RNA molecule is recognized by the RNA-induced silencing complex, which unwinds the duplex and allows for the antisense strand to be reutilized to form more dsRNA for Dicer. The initially cleaved mRNA (not part of the duplex) is degraded by RNAse within the cell, causing the null phenotype. Although this mechanism is thought to normally regulate gene expression, the artificial addition of chemically synthesized antisense RNA can mediate the phenomenon. RNAi has been shown to work in humans, mice, worms, insects, and in our proposed context—fish (Boonanuntanasarn et al. 2003). A slight disadvantage is that the phenotype is not 100% reduced. Often, the expression is lowered by 80–95%, as determined by Northerns/Westerns. Recently, TG siRNAs are the new players on the block (Kunach et al. 2003). Often, if linear DNA is used, the expression is stable and lasts for the life of animal. If injected into fertilized cells at the single-cell stage, the injected DNA can often show germline transmission. To increase the efficiency of transfection of the siRNA, vectors are being inserted into defective viruses.

CONCLUSIONS

Our goal is to produce the ideal donor tissue for clinical islet xenotransplantation. Clinical islet xenotransplantation, for the foreseeable future, could only be accomplished using encapsulated islet grafts. According to Dionne et al. (1994), the ideal islet tissue for encapsulation would be one that has a high insulin output, is correctly regulated by glucose and other secretogogues, has low metabolic demand, and is capable of functioning for extended periods without replacement. In addition, the cells must be procurable in high yield at reasonable cost with protocol meeting FDA standards. Tilapia islets meet many of these expectations; in fact, they are extremely well suited for encapsulation relative to mammalian islets because they tolerate exceedingly low oxygen tensions without loss of function or viability. Tilapia islets produce tilapia insulin, which differs from human insulin by 17 amino acids, and, thus, would likely have diminished activity and may

be immunogenic. To help circumvent these problems, we have produced TG tilapia with islets that secrete humanized insulin. One of the major drawbacks of using islets from our TG tilapia is that their islets also still produce tilapia insulin. Therefore, after breeding our TG tilapia to achieve homozygocity, we hope to develop the technology to either KO or silence the production of tilapia insulin so that we have donors that produce only humanized insulin.

The availability of a simple, inexpensive source of islets would likely revolutionize clinical islet transplantation and could be the elusive "cure" for type I diabetes. Interestingly, we are not the first to suggest that fish islets could be used in the treatment of clinical diabetes mellitus, as Prof. JJR McLeod, Banting's and Best's mentor and one of the discoverers of insulin, very aggressively pursued the concept of using BBs as a source of insulin in the early 1920s (Wright 2002a). Perhaps, he was on to something.

REFERENCES

Alexander E, Dooley KC, Pohajdak B, and Wright JR Jr. 2006. Things we've learned from tilapia islet xenotransplantation. Gen Com Endocrinol. 148: 125–131.

Armstrong D. 2003. Patrick Soon-Shiong is tantalizingly close to a cancer breakthrough with huge implications. So why do so many people want to see him fail? The Richest People in America. Forbes Magazine. October 6. http://www.forbes.com/forbes. 2003/1006/126.

Boonanuntanasarn S, Yoshizaki G, and Takeuchi T. 2003. Specific gene silencing using small interfering RNAs in fish embryos. Biochem Biophys Res Com. 310: 1089–1095.

Dionne K, Scharp D, Lysaght M, Hegre O, and Lacy P. 1994. Macroencapsulation of islets for the treatment of diabetes. In: Pancreatic Islet Transplantation. Vol III. Immunoisolation of Pancreatic Islets (eds. Lanza RP and Chick WL). RG Landes, Austin, TX pp 119–131.

Fishman JA and Patience C. 2004. Xenotransplantation: infectious risk revisited. Am J Transplant. 4: 1383–1390.

Hong Y, Chen S, Gui J, and Schartl M. 2004. Retention of the developmental pluripotency in medaka embryonic stem cells after gene transfer and long-term drug selection for gene targeting in fish. Transgenic Res. 13: 41–50.

Kawakami K and Hopkins N. 1996. Rapid method of screening transgenic zebrafish. TIG. 12: 9–10.

Kelly KM. 1993. Experimental diabetes mellitus in a teleost fish. I. Effect of complete isletectomy and subsequent hormonal treatment on metabolism in the goby, *Gillichthys mirabilis*. Endocrinol. 132: 2689–2695.

Kunach T, Gish G, Lickert H, Jones N, Pawson T, and Rossant J. 2003. Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nature Biotechnol. 21: 559–561.

Leventhal J, Kissler H, Xu B-Y, Xia G, Kaufman D, and Wright JR Jr. 2006. Piscine Islets Can Reverse Hyperglycemia in Diabetic Nonhuman Primates. World Transplant Congress, Boston, MA.

MacNeil SD. 2002. The development of tilapia (*Oreochromis niloticus*) chimeras and short-term culture of undifferentiated embryo cells. M.Sc. Thesis. Dalhousie University, Pathology Department.

Mansour M, Wright JR Jr., and Pohajdak B. 1998. Cloning, sequencing and characterization of the tilapia insulin gene. Comp Biochem Physiol. 121B: 291–297.

McManus MT and Sharp PA. 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet. 3: 737–747.

Morrison CM, Miyake T, and Wright JR Jr. 2001. Histological study of the development of embryo and early larva of *Oreochromis niloticus* (Pisces: Cichlidae). J Morphol. 247: 172–195.

Morrison CM, Pohajdak B, and Wright JR Jr. 2003. Structure and enzymatic removal of the chorion of embryos of the Nile tilapia *Oreochromis niloticus*. J Fish Biol. 63: 1439–1453.

Morsiani E, Lebow LT, Rozga J, and Demetriou AA. 1995. Teleost fish islets: a potential source of endocrine tissue for the treatment of diabetes. J Surg Res. 58: 583–591.

Muller U. 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech Dev. 82: 3–21.

Nguyen T, Wright JR Jr., Nielsen PF, and Conlon JM. 1995. Characterization of the pancreatic hormones from the Brockmann body of the tilapia—implications to islet xenograft studies. Comp Biochem Physiol. 111C: 33–44.

Patience C, Takeuchi Y, and Weiss RA. 1997. Infection of human cells by an endogenous retrovirus of pigs. Nature Med. 3: 282–286.

Pohajdak B, Mansour M, Hrytsenko O, Conlon JM, Dymond C, and Wright JR Jr. 2004. Production of transgenic tilapia with Brockmann bodies secreting [desThrB30] human insulin. Transgenic Res. 13(4): 313–323.

Rahman MA and MacLean N. 1992. Production of transgenic tilapia (*Oreochomis niloticus*) by one-cell stage microinjection. Aquaculture 105: 219–232.

Rayat GR, and Gill RG. 2003. Pancreatic islet xenotransplantation: barriers and prospects. Cur Diab Rep. 3: 336–343.

Ricordi C, Socci C, Davalli AM, Staudacher C, Baro P, Vertova A, Sassi I, Gavazzi F, Pozza G, and Di Carlo V. 1990. Isolation of the elusive pig islet. Surgery 107: 688–694.

Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, and Shapiro AMJ. 2005. Five-year follow-up after clinical islet transplantation. Diabetes 54: 2060–2069.

Shapiro AMJ, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, and Rajotte RV. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med. 343: 230–238.

Shears MA, King MJ, Goddard SV, and Fletcher GL. 1992. Gene transfer in salmonids by injection through the micropyle. In: Transgenic Fish (eds. Hew CL and Fletcher GL), World Scientific, New Jersey. pp 44–60.

Sihvonen L. 2004. Xenotransplantation and the potential threat of animal pathogens in humans. Acta Vet Scand Suppl. 99: 35–37.

Simon SG. 1996. VivoRx's diabetes cell therapy product granted orphans drug status and IND approval; encapsulated porcine cell therapy product aims to meet supply for donor islets in battle against diabetes. Business Wire. July 22. http://findarticles.com/p/articles/mi_m0EIN/is_/ai_18500345.

Wilson CA. 2008. Porcine endogenous retroviruses and xenotransplantation. Cell Mol Life Sci. 65: 3399–3412.

Wright JR Jr. 1992. Experimental transplantation using principal islets of teleost fish (Brockmann bodies). In: Pancreatic Islet Cell Transplantation: l892–l992—One Century of Transplantation for Diabetes (ed. Ricordi C). RG Landes, Austin. pp 336–351.

Wright JR Jr. 1994. Procurement of fish islets (Brockmann bodies). In: Pancreatic Islet Transplantation Series. Vol. l: Procurement of Pancreatic Islets (eds. Lanza RP and Chick WL). RG Landes, Austin, TX pp l23–133.

Wright JR Jr. 2002a. From ugly fish to conquer death: JJR Macleod's fish insulin research, 1922–24. Lancet. 359: 1238–1242.

Wright JR Jr. 2002b. Alternative interpretations of the same data: flaws in the process of consulting the Canadian public on xenotransplantation issues. CMAJ. 167: 40–42.

Wright JR Jr. 2004. Public consultation on xenotransplantation. Transplantation 78: 1112–1113.

Wright JR Jr. 2006. Academic uses of GM fish technology: a call for a common sense approach to regulation. In: Assessment of Environmental and Indirect Human Health Effects of Genetically Modified Aquatic Organism (ed. Devlin RH). Canadian Technical Report of Fisheries and Aquatic Sciences 2581. Department of Fisheries & Oceans Canada, Ottawa. 111–126.

Wright JR Jr. and Pohajdak B. 2000. Transgenic fish and a method of harvesting islet cells therefrom. US Patent No. 6015713. Issued January 18, 2000.

Wright JR Jr. and Pohajdak B. 2001. Cell therapy for diabetes using piscine islet tissue. Cell Transplant. 10: 125–143.

Wright JR Jr. and Pohajdak B. 2002. Transgenic fish in the treatment of diabetes. U.S. Patent No. 6476290B1. Issued November 5, 2002.

Wright JR Jr., Pohajdak B, Xu B-Y, and Leventhal JR. 2004. Piscine islet xenotransplantation. ILAR J. 45: 314–323.

Wright JR Jr., Polvi S, and MacLean H. 1992. Experimental transplantation using principal islets of teleost fish (Brockmann bodies): long-term function of tilapia islet tissue in diabetic nude mice. Diabetes. 41: 1528–1532.

Wright JR Jr., Snowden J, Hrytsenko O, Morrison CM, and Pohajdak B. 2008. Immunohistochemical staining for tilapia and human insulin demonstrates that a tilapia transgenic for humanized insulin is a mosaic. Transgenic Res. 17: 991–992.

Wright JR Jr., Yang H, and Dooley KC. 1998. Tilapia—a source of hypoxia-resistant islets for encapsulation. Cell Transplant. 7: 299–307.

Yang H, Dickson B, O'Hali W, Kearns H, and Wright JR Jr. 1997a. Functional comparison of mouse, rat, and fish islet grafts transplanted into diabetic nude mice. Gen Comp Endocrinol. 106: 384–388.

Yang H, O'Hali W, Kearns H, and Wright JR Jr. 1997b. Long-term function of fish islet xenografts in mice by alginate encapsulation. Transplantation. 64: 28–32.

Yang H and Wright JR Jr. 1995. A method for mass harvesting islets (Brockmann bodies) from teleost fish. Cell Transplant. 4: 621–628.

Yang H and Wright JR Jr. 2002. Alginate encapsulation (Ca^{+2}-induced gelation). In: Methods of Tissue Engineering (eds. Atala A and Lanza R). Academic Press, San Diego. pp 787–801.

Chapter 19

The Potential of Enhancing Muscle Growth in Cultured Fish through the Inhibition of Members of the Transforming Growth Factor-β Superfamily

Michael P. Phelps and Terence M. Bradley

INTRODUCTION

Growth and differentiation of muscle in mammals is regulated by several transforming growth factor-β (TGF-β) superfamily members, including myostatin. However, it remains unclear whether TGF-β molecules perform a similar function in fish. Natural mutations in the myostatin gene in some breeds of cattle, and myostatin knockout or inhibition in mice, cause marked increases in muscle mass (Kambadur et al. 1997; McPherron et al. 1997; Lee and McPherron 2001). The potential of increasing muscle mass in cultured fish through inhibition of myostatin or other TGF-β molecules is now being realized, with the recent production of transgenic rainbow trout (*Oncorhynchus mykiss*) overexpressing the broad TGF-β inhibitor, follistatin (Medeiros et al. 2009). In this chapter, we discuss the possible mechanisms by which members of the TGF-β superfamily regulate muscle growth in fish and how these pathways might be manipulated to enhance the growth of commercially valuable species.

MYOSTATIN DEFICIENCY AND INHIBITION

Several examples of naturally occurring myostatin-deficient animals exist. The most well-known of these are double muscled cattle (e.g., Piedmontese and Belgian Blue breeds), which have loss of function of the myostatin gene. The mutation found in the Piedmontese myostatin gene converts an invariant cysteine to a tyrosine at amino acid 313 (C313Y mutation) (Berry et al. 2002). The myostatin gene in Belgian Blue cattle has an 11 nucleotide deletion that introduces a frame shift and truncation of the protein at amino acid 288 (Kambadur et al. 1997; McPherron and Lee 1997). Belgian Blue and Piedmontese cattle have approximately 20% more muscle mass, a reduction in body fat, and enhanced feed

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

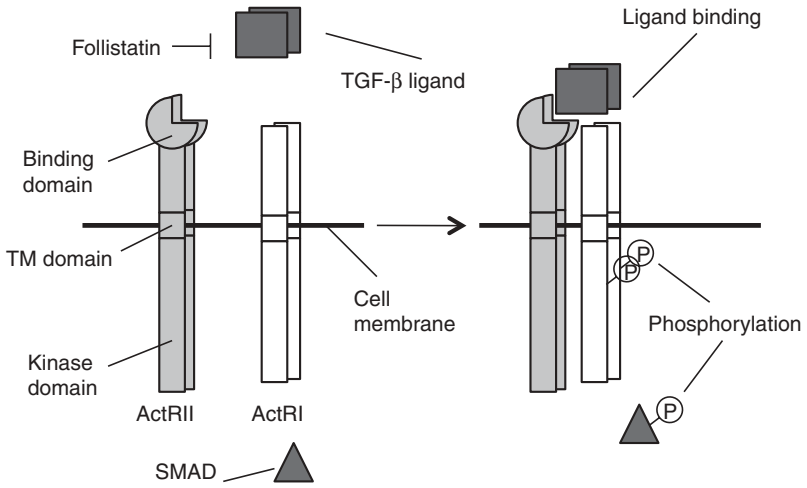


Figure 19.1. The activin receptor signaling pathway utilized by several TGF-β ligands including myostatin. TGF-β ligands bind to the extracellular domain of the activin type II receptor (ActRII), which then recruits and phosphorylates the activin type I receptor (ActRI). Phosphorylated ActRI propagates TGF-β signal transduction by activation of a SMAD signaling cascade. The extracellular, transmembrane (TM), and intracellular kinase domains of both activin receptors are identified.

conversion efficiency (Kambadur et al. 1997; Casas et al. 1998).

Laboratory-generated mice lacking myostatin (i.e., knockout) exhibit a significant increase in both muscle fiber hypertrophy and hyperplasia and develop twice the muscle mass of wild-type mice (McPherron et al. 1997). Myostatin-null mice also have a decrease in the percentage of intermuscular fat and connective tissue. The reduction in fat accumulation may be associated with a decrease in leptin levels (Lin et al. 2002) and an increase in metabolic rate, and insulin sensitivity in muscle fibers (Guo et al. 2009).

Transforming Growth Factor-β Muscle Regulatory Pathway

The transforming growth factor β superfamily comprised an array of proteins, including myostatin, activins, growth and differentiation factors (GDF) 9 and 11, and bone morphogenetic proteins. Signal transduction occurs via coordinated binding and activation of two serine threonine kinase receptors located on the cell membrane of target tissues. Myostatin and several other TGF-β ligands signal through the activin type I and type II transmembrane receptors (Link and Nishi 1997; Lee et al. 2005). Type II receptors bind TGF-β ligands, causing recruitment and hyperphosphorylation of type I receptors (Attisano et al. 1996; Fig. 19.1). Phosphorylated type I receptors then propagate TGF-β signals by activation of a SMAD signaling cascade (Attisano and Wrana 2002; Fig. 19.1). While the activin type II receptors (ActRIIA and ActRIIB) are utilized by numerous TGF-β ligands, signaling specificity is maintained through various combinations of SMAD and type I receptor interactions (Feng and Derynck 2005). Activin type II and type I receptors interact through the intracellular serine/threonine kinase domains of each receptor and truncation of the type II receptor kinase domain has been used to inhibit signal transduction (Tsuchida et al. 2008).

The Myostatin Protein

The myostatin polypeptide, like many of the TGF-β superfamily members, contains three distinct domains, the N-terminal signaling domain, the

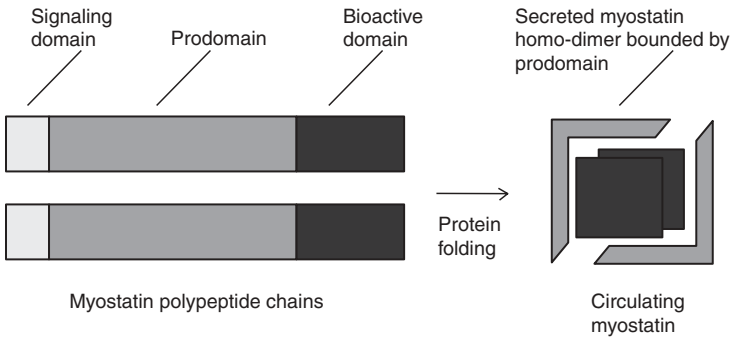


Figure 19.2. The myostatin peptide consists of three domains: the N-terminal signaling domain, the prodomain, and the bioactive domain. Each myostatin domain is cleaved by posttranslational processing. The myostatin prodomain is a major inhibitor of circulating myostatin.

prodomain, and the C-terminal bioactive domain (Lee and McPherron 2001; Fig. 19.2). Nine conserved cysteine residues in the active domain of the myostatin peptide stabilize the tertiary structure and form a disulfide-linked homodimer (Cash et al. 2009).

Myostatin is secreted in a noncovalently bound latency complex with the myostatin prodomain (Lee and McPherron 2001; Anderson et al. 2008; Fig. 19.2). Formation of the latency complex is one of the primary regulators of myostatin activity in vivo and may account for more than 70% of circulating myostatin (Hill et al. 2002). Myostatin is also bound by follistatin (Fig. 19.1), which inhibits multiple TGF-β ligands, including activin and GDF-11 (Welt et al. 2002; Liu 2006). Other myostatin antagonists include the follistatin-related gene, the human small glutamine-rich tetratricopeptide repeat-containing protein, the titin-cap, and the GDF-associated serum protein-1.

Inhibition of Myostatin and Other TGF-β in Mammals

Inhibition of myostatin in mammals has been attained by overexpression of the myostatin prodomain (Lee and McPherron 2001), overexpression of a myostatin molecule lacking the proteolytic cleavage site (Zhu et al. 2000), and antibodies to myostatin (Whittemore et al. 2003). Enhanced muscle growth similar to or exceeding that seen with inhibition of myostatin has been accomplished through the use of broad TGF-β inhibitors, such as a truncated or soluble ActRIIB, follistatin, or the follistatin-related gene. Indeed, muscle mass can be quadrupled in mice by overexpressing follistatin in the muscle tissue of myostatin null mice (Lee 2007). Injection of soluble ActRIIB also increases the muscle mass of myostatin null mice (Lee et al. 2005), suggesting that myostatin is not the sole muscle regulatory factor in mammals, since several TGF-β ligands use the ActRIIB and are inhibited by follistatin.

It remains unclear what factors regulate mammalian muscle growth in addition to myostatin. Other than myostatin, only GDF-11 and activins A, B, and AB utilize the ActRIIB to inhibit myoblast differentiation in vitro (Souza et al. 2008). GDF-11 has high sequence similarity to myostatin and, like myostatin, is inhibited by follistatin (Lee 2004; Lee et al. 2005). Studies in mice, however, suggest that GDF-11 does not regulate muscle growth (McPherron et al. 2009).

MYOSTATIN AND FISH

The myostatin gene has been identified in over 55 species of fish, and the peptide sequence of the bioactive domain is highly conserved (circa 89%) between fish and terrestrial vertebrates. In contrast to mammals, which have a single myostatin gene (McPherron et al. 1997), many species of fish possess multiple forms of myostatin due to genome duplication events. Phylogenetic analysis suggests that the majority of osteichthyan species possess at least two myostatin genes with salmonids having four (Rodgers et al. 2007). Myostatin is also ubiquitously expressed throughout the tissues of fish

(Garikipati et al. 2006), unlike in mammals where it is largely restricted to muscle. These findings suggest that myostatin may have functions in fish in addition to inhibiting the growth of muscle tissue.

Muscle Growth in Fish

The development and growth of muscle tissue in fish differs from that of terrestrial vertebrates. Fish muscle consists of segmentally arranged myotomes in which slow and fast muscle fibers are physically separated (Johnston 2006). Fish are also able to recruit new muscle fibers into adulthood (hyperplasia) in addition to increasing the size (hypertrophy) of existing muscle fibers (Johnston 2006). Fish species that are limited in size (i.e., exhibit determinate growth) increase muscle fiber number through stratified hyperplasia, which recruits new muscle fibers along specific germinal layers (Rescan 2005). Large fish species exhibit indeterminate growth and are able to recruit new muscle fibers throughout the musculature (i.e., mosaic hyperplasia) in addition to stratified hyperplasia (Rescan 2005).

Myostatin and TGF-β Inhibition in Fish

The enhanced muscle growth achieved by inhibition of myostatin or other TGF-βs in mammals has stimulated widespread research aimed at determining the function of TGF-βs in teleost fish. Increases in weight have been reported with overexpression of the myostatin prodomain and with RNA interference in zebrafish (Xu et al. 2003; Acosta et al. 2005; Lee et al. 2009). Immersion of 0.9 g rainbow trout in recombinant myostatin prodomain protein has been reported to increase the weight of fish, but uptake of the protein was not demonstrated and the study was only conducted for a short period of time (Lee et al 2010). Injection or immersion of a soluble ActRIIB protein was reported to increase the size of juvenile and larval goldfish (*Carassius auratus*), African catfish (*Clarias gariepinus*), and tilapia (*Oreochromis aureus*; Carpio et al. 2009). As above, the research was conducted over a short duration and with small fish. In contrast, overexpression of a dominant negative myostatin molecule in transgenic medaka failed to promote muscle growth (Sawatari et al. 2010). Although several of these reports might suggest that TGF-βs regulate muscle growth in larval fish, the duration of the studies and absence of evidence demonstrating uptake leaves questions, particularly whether similar results might be anticipated in larger fish over an extended period of time.

Of these investigations, only three examined the inhibition of myostatin/TGF-βs in adult animals, and all employed zebrafish or medaka as model organisms (Xu et al. 2003; Acosta et al. 2005; Lee et al. 2009). The determinate growth of zebrafish and medaka limits their utility in deciphering mechanisms regulating muscle growth in large, commercially valuable fish species (e.g., rainbow trout), which exhibit indeterminate growth.

On a related note, a GDF-11 ortholog has been discovered in zebrafish (*Danio rerio*; NM_212975; Biga et al. 2005) and rainbow trout (*O. mykiss*; HM143890). However, it remains uncertain whether GDF-11 is involved in regulating muscle growth in fish.

PRODUCTION OF TRANSGENIC TROUT OVEREXPRESSING FOLLISTATIN

Rainbow Trout as a Model Organism

To gain insight into whether TGF-β superfamily members influence muscle growth in fish species that exhibit indeterminate growth, transgenic rainbow trout have been produced overexpressing the broad TGF-β inhibitor, follistatin. Using a widely cultured species, rainbow trout, as a model for investigating TGF-β signaling inhibition ensured that information gained would be broadly applicable to the aquaculture industry. Another benefit of using rainbow trout was the availability of significant molecular data and established husbandry (Piper et al. 1982) and microinjection techniques (Du et al. 1992). Transgenic organisms were produced to investigate the phenotypic effect of follistatin overexpression throughout the lifespan of rainbow trout as well as to potentially produce stable, growth-enhanced, transgenic lines. These criteria are important for both basic and applied research.

Follistatin Construct

To limit undesired phenotypic effects, a muscle-specific expression vector (*mylc*) was used to express follistatin in the muscle tissue of transgenic fish. The complete rainbow trout follistatin coding sequence

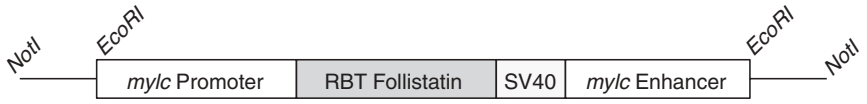


Figure 19.3. Schematic of the *mylc* expression vector carrying the rainbow trout follistatin coding sequence (CDS). The rainbow trout follistatin CDS was inserted downstream of the *mylc* promoter and upstream of a SV40 polyadenylation sequence and the *mylc* enhancer. The locations of *EcoRI* and *NotI* restriction sites used to linearize the follistatin gene construct in preparation for microinjection are indicated.

(CDS; Accession: FJ185129) was cloned into the *mylc* plasmid vector on the 3' end of a rat myosin light chain promoter (Rosenthal et al. 1989; Fig. 19.3). An SV40 polyadenylation sequence and the rat myosin light chain enhancer are located downstream of the 5' end of the follistatin CDS (Fig. 19.3). A reporter construct also was produced by replacing the follistatin CDS with the CDS of the green fluorescent protein (GFP). This construct was used to verify translation and tissue distribution of *mylc* driven expression in rainbow trout. To prepare the constructs for microinjection, the plasmids were cleaved at two locations with a NotI restriction enzyme to linearize the construct and to remove the remainder of the vector backbone (Fig. 19.3). Digested plasmids were purified using agarose gel electrophoresis.

Microinjection and Transgenic Screening

Rainbow trout eggs from the Shasta and Arlee strain (Ennis National Fish Hatchery, Ennis, MT) were fertilized 30 minutes prior to microinjection and maintained in 4°C rainbow trout isotonic saline (9.04 g/L NaCl, 0.24 g/L KCl, 0.34 g/L $CaCl_2$) to prevent hardening of the chorion and to slow development. Eggs were injected with a pulled glass pipette (World Precision Instruments, Inc. Sarasota FL), which was attached to a 5 μL positive displacement syringe (Hamilton Company, Reno, NV) with surf wax (Mr. Zog's Sex Wax, Carpinteria, CA). One million copies of the follistatin or GFP construct (5 nL of 200,000 copies/nL) was microinjected through the micropyle of each egg. To visualize the site of injection, each construct was diluted in 4% green food coloring. After injection, the eggs were maintained in flow-through incubators (Heath Tecna Corp., Kent, WA) until hatched.

Identifying the limited number of P1 transgenic organisms is a major logistical challenge when producing transgenics by microinjection. To screen microinjected larvae for the presence of the construct, a small piece of caudal fin tissue was excised using a dissecting scalpel and placed in a 0.5 mL microcentrifuge tube containing 200 μL of lysis buffer (50 mmol/L KCl, 10 mmol/L Tris pH 8.8, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100). The tissue was then boiled for 5 minutes, followed by digestion with proteinase K (Sigma-Aldrich, St. Louis, MO) at 55°C for 1 hour. After centrifuging the samples at 12,000 *g* for 5 minutes, the DNA from 2 μL of supernatant was amplified in a PCR reaction containing a *mylc* promoter-specific forward primer and a follistatin-specific reverse primer. Transgenic fish were identified by the presence of an amplicon on a 1% agarose gel (Fig. 19.4). When detecting low

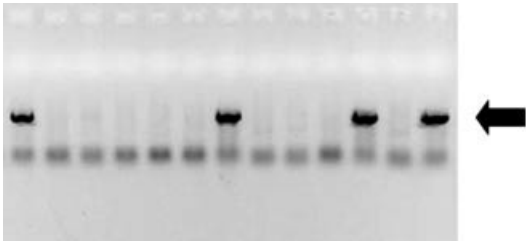


Figure 19.4. Photo of agarose gel showing PCR amplicons from transgenic fish (black arrow). Samples lacking an amplicon are negative fish that did not contain the construct. Modified figure originally published by Medeiros et al. (2009) and was used with permission from the *American Journal of Physiology*.

Table 19.1. Production Statistics for Rainbow Trout Eggs Microinjected with the Follistatin DNA Construct.

	Groups of Microinjected Eggs		
	Group 1	Group 2	Group 3*
Number of eggs microinjected	1863	1047	1837
Number of surviving juveniles	319	321	676
Number of transgenic fish	1	13	171
Percent survival	17%	31%	37%
Rate of transgenesis	0.3%	4%	26%

*Group 3 transgenic fish were identified by the presence of the "six pack" phenotype, rather than PCR, which could have underestimated the number of transgenic organisms. To verify the rate of transgenesis, a random subsample of individuals from the group 3 population (transgenic and nontransgenic) was subsequently screened by PCR and 26% of these fish possessed the transgene.

rates of transgenesis, fin clips from 3–5 individuals were pooled for each sample, and positive samples were rescreened individually to identify transgenic individuals.

Of the three groups microinjected with the follistatin construct, one (group 3) had a particularly high percentage of transgenic organisms (26%; Table 19.1). Fish from group 3 enabled characterization of the P1 generation since it provided sufficient transgenic organisms for examination of muscle physiology, while maintaining the individuals for broodstock.

FOLLISTATIN OVEREXPRESSION IN TROUT INFLUENCES MUSCLE GROWTH

Follistatin Overexpression

Follistatin transcript levels in P1 control and transgenic fish from group 3 were measured using quantitative reverse transcription PCR to deduce relative levels of construct expression in P1 transgenic fish. Transgenic fish had follistatin transcript levels 18-fold higher than controls (follistatin transcript, mean $\pm$ SE in 50 ng of total RNA: transgenic ($n = 5$), 43,117 $\pm$ 23,593; control ($n = 5$), 2422 $\pm$ 422; Fig. 19.5A). Follistatin expression varied widely among transgenic individuals with one fish having follistatin transcript levels 21 times (901,900 transcripts) greater than the average transgenic fish (43,117 transcripts). Transgenic fish expressing the GFP reporter had high levels of GFP expression in muscle tissue, suggesting that the *mylc* vector is capable of driving expression in the muscle tissue of rainbow trout (Fig. 19.6).

Localized Muscle Growth in P1 Follistatin Transgenic Fish

A prominent phenotype developed in P1 follistatin transgenic fish characterized by localized regions of muscle growth in both the epaxial and hypaxial musculature (Fig. 19.7). The phenotype was dubbed "six pack" because of its similarity to well-defined rectus abdominus and intercostal muscles in humans. The localized regions of muscle growth appeared to increase with the age of the fish, suggesting an enhanced rate of muscle growth in these areas (Fig. 19.8). Several fish also exhibited enlarged jaw abductor muscles (Fig. 19.8).

Muscling in transgenic fish was readily apparent along the inner abdominal wall and was often confined to specific myotomes (Fig. 19.9). The phenotype was not caused by rippling or flexing of the musculature but by defined regions of enhanced muscle growth (Fig. 19.9). The magnitude and location of muscling varied between individuals and several fish exhibited asymmetrical muscle growth.

It is currently unclear why P1 transgenic fish exhibited localized regions of muscle growth, since the axial musculature of teleost fish lack defined muscle groups. It is possible that the phenotype was induced by differential follistatin expression among myotomes or by a mosaic integration of the DNA construct in muscle tissue of the P1 generation. Mosaicism develops when genomic integration of the DNA construct occurs at a late stage of development, causing only a percentage of cells in a given tissue to incorporate and express the transgene. Somatic and germ tissue mosaicism is common for both fish and

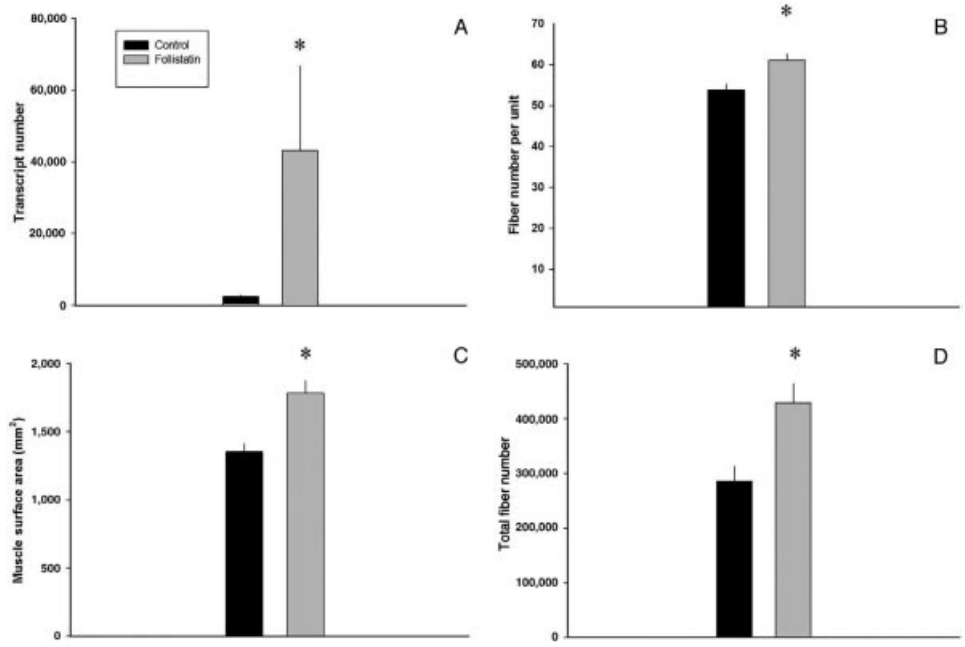


Figure 19.5. Follistatin transcript levels in 50 ng of RNA from transgenic and control RBT (**A**). The number of muscle fibers per 0.253 mm^2 unit area (**B**). Average total cross-sectional surface area (**C**). Number of muscle fibers per cross section (**D**). Modified graphs originally published by Medeiros et al. (2009) and were used with permission from the *American Journal of Physiology*.

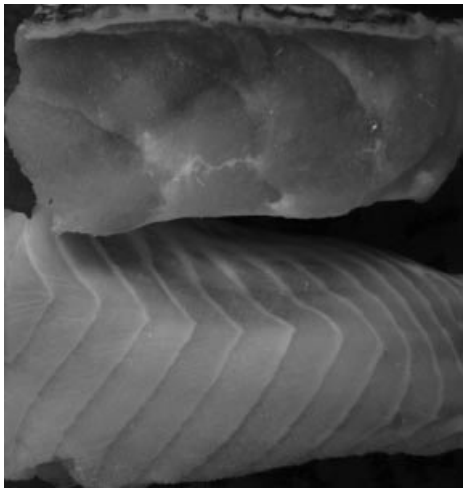


Figure 19.6. GFP protein synthesis in the muscle tissue of a GFP transgenic fish (bottom). No GFP was detected in the muscle tissue of control fish (top). (For color detail, please see the color plate section.)

mammalian P1 transgenic organisms (Stuart et al. 1990; Gross et al. 1992; Whitelaw et al. 1993; Rahman et al. 2000). The "six pack" phenotype has not been observed in preliminary data from the F1 generation, suggesting that the phenotype is an artifact of producing P1 transgenic organisms and that any growth-enhanced transgenic lines should have muscle growth throughout the axial musculature (i.e., no localized muscle growth).

In addition to the "six pack" phenotype, P1 transgenic fish exhibited a significant increase in condition factor. Condition factor is a ratio of weight (W in grams) to length (L in centimeters) ($(W/L^3) \times 100$), with a large condition factor representing a more compact body morphology, e.g., greater girth. The significant increase in condition factor exhibited by transgenic fish suggests that follistatin overexpression stimulated a marked change in the pattern of muscle growth. It appears likely that muscle growth may outpace linear growth of the skeletal system in the follistatin transgenic fish.

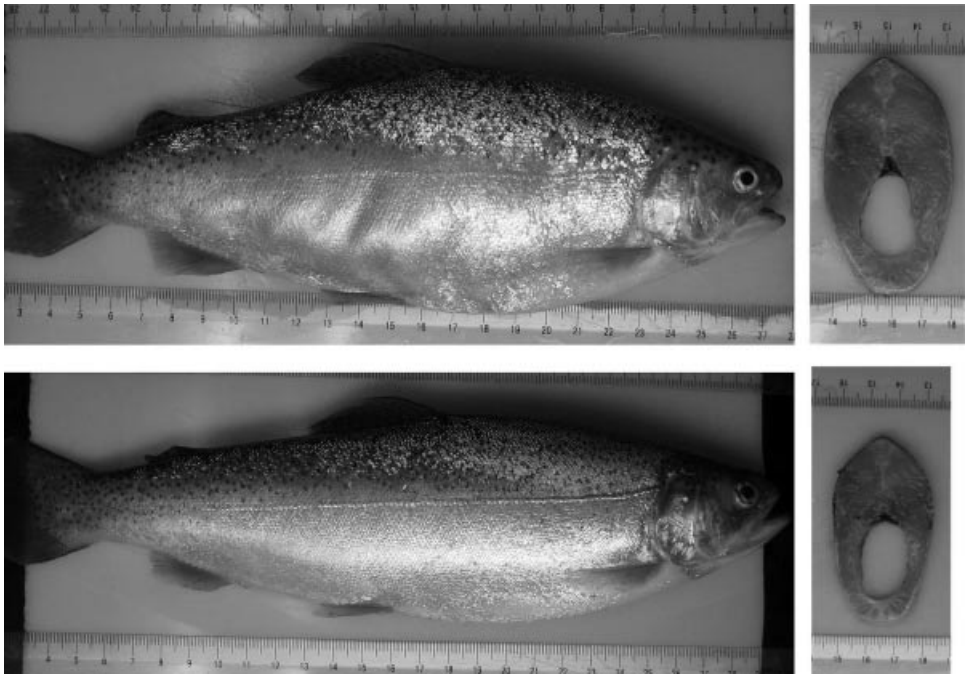


Figure 19.7. The "six pack" phenotype observed in P1 juvenile transgenic fish overexpressing follistatin (top). Control trout (bottom) did not exhibit any enhanced muscling. Cross sections from a subsample of transgenic and control fish were used to quantify total cross-sectional surface area and to extrapolate the total number of muscle fibers per cross section. Modified figure originally published by Medeiros et al. (2009) and was used with permission from the *American Journal of Physiology*.

Figure 19.8. A 2-year-old P1 transgenic fish with a prominent "six pack" phenotype. The localized regions of muscle growth are enhanced with age and some fish exhibit increased growth of the jaw abductor muscles.

Hyperplasia of Muscle Fibers in Transgenic Fish

Muscle histology performed on a subsample of P1 juvenile transgenic and control fish revealed muscle fiber hyperplasia in transgenic fish (Fig. 19.5). The number of muscle fibers in six replicate 0.253 mm^2 subsections was quantified for both P1 transgenic and control trout and total cross sectional surface area was used to extrapolate the total number of fibers per cross section. Transgenic fish had an average of 61 ± 1.6 muscle fibers per unit area compared to 53.8 ± 1.4 in control fish ($p < 0.05$; Fig. 19.5B). Transgenic fish also had a larger number of muscle fibers per cross section, which was due in part to an increase in the total cross-sectional surface area of transgenic fish ($p < 0.05$; Figs. 19.5C and 19.5D).

The average diameter of muscle fibers in transgenic fish was significantly less than that of

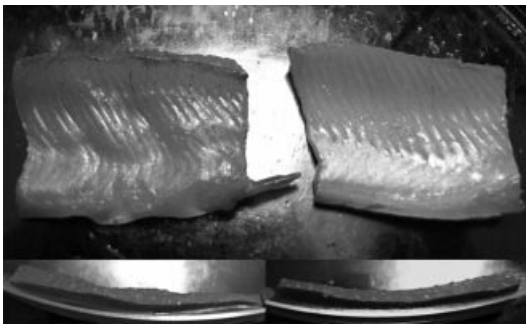


Figure 19.9. The inner abdominal wall of a juvenile P1 follistatin transgenic fish (top left) compared to a control fish (top right). Increased muscle growth is observed in defined regions of the musculature in P1 transgenic fish and often obscures the bones of the abdominal wall. The abdominal wall of control fish exhibit uniform muscle development and the bone are clearly visible. Rough cross sections of the abdominal wall of P1 transgenic (bottom left) and control fish (bottom right) suggest that enhanced musculature may be caused by localized muscle growth.

controls, and there was a marked shift in the overall distribution of muscle fibers (Fig. 19.10). This evidence suggests that the muscle growth observed in juvenile P1 transgenic fish resulted from an increase in the proliferation of muscle fibers. Further research is required to determine whether follistatin overexpression stimulates hyperplastic muscle growth in all developmental stages (i.e., larval and adult) or if the mechanisms of muscle growth differ throughout ontogeny.

Transgenic F1 Offspring and the Future of Follistatin Overexpressing Transgenic Trout

The P1 transgenic generation provides insight into the phenotypic effects of follistatin overexpression in rainbow trout, but full characterization requires stable transgenic lines. Mendelian inheritance has not been observed in F1 lines obtained to date, suggesting that P1 founder organisms were mosaic in germ tissue. This germline mosaicism reduced the number of F1 transgenic offspring produced in the first spawning season. This in combination with the large variability in the growth of F1 transgenic organisms limits conclusive characterization of F1 growth at this time. There is, however, preliminary evidence of a compact, more muscular phenotype in F1 transgenic individuals. Although these rainbow trout provide an excellent model for investigating the mechanisms by which TGF-β ligands regulate muscle growth in fish, the lengthy time required to attain sexual maturation (2 years of age) and single annual spawning period requires a long-term investment to generate consistent transgenic lines.

DISCUSSION

Piscine Muscle Growth is Regulated by TGF-β Signaling

The phenotype observed with overexpression of follistatin in rainbow trout indicates that myostatin or related TGF-β molecules are involved in regulating muscle growth and development in fish. It is unclear, however, exactly which TGF-β ligands are responsible for causing the "six pack" phenotype or the compact body morphology exhibited in P1 transgenic fish, since follistatin inhibits numerous TGF-β superfamily members. Research is ongoing to understand the molecular mechanisms behind the phenotype and to investigate alternative methods of inhibiting TGF-β signaling in muscle tissue. While it is apparent that increased muscling and changes in morphology result from inhibiting TGF-β signaling in muscle tissue, it is still unclear whether stable transgenic lines overexpressing follistatin will exhibit overall enhanced muscle growth. Further investigation of the transgenic fish described herein will be instrumental to understanding which factors regulate the growth of muscle tissue in fish and how these signaling pathways can be manipulated to improve aquaculture production worldwide.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of Erika Medeiros, Marc Concepcion, Fernando Fuentes, Ian Jaffe, and Tori Spence, who were involved in the production and characterization of the follistatin transgenic fish. The research presented here was supported by National Research Initiative

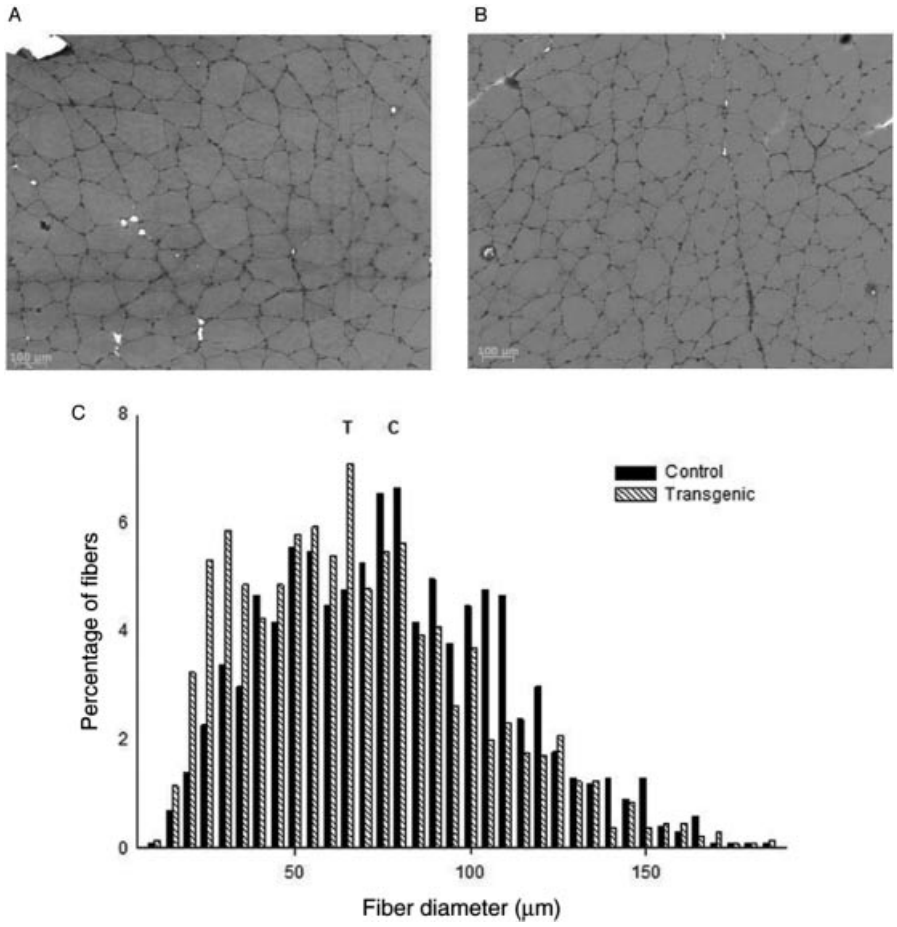


Figure 19.10. Representative muscle sections from control (**A**) and transgenic (**B**) fish. Fiber diameter distribution from transgenic and control fish (**C**). Graph and figures originally published by Medeiros et al. (2009) were used with permission from the *American Journal of Physiology*.

Competitive Grant no. 2009-35206-05288 from the USDA National Institute of Food and Agriculture and by the Rhode Island Agriculture Experiment Station.

REFERENCES

Acosta J, Carpio Y, Borroto I, Gonzalez O, and Estrada MP. 2005. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol. 119: 324–331.

Anderson SB, Goldberg AL, and Whitman M. 2008. Identification of a novel pool of extracellular promyostatin in skeletal muscle. J Biol Chem. 283: 7027–7035.

Attisano L and Wrana JL. 2002. Signal transduction by the TGF-β superfamily. Science. 296: 1646–1647.

Attisano L, Wrana JL, Montalvo E, and Massague J. 1996. Activation of signaling by the activin receptor complex. Mol Cell Biol. 16: 1066–1073.

Berry C, Thomas M, Langley B, Sharma M, and Kambadur R. 2002. Single cysteine to tyrosine transition inactivates the growth inhibitory function of Piedmontese myostatin. Am J Physiol Cell Physiol. 283: C135–C141.

Biga PR, Roberts SB, Iliev DB, McCauley LAR, Moon JS, Collodi P, and Goetz FW. 2005. The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, *Danio rerio*. Comp Biochem Physiol B Biochem Mol Biol. 141: 218–230.

Carpio Y, Acosta J, Morales R, Santisteban Y, Sanchéz A, and Estrada MP. 2009. Regulation of body mass growth through activin type IIB receptor in teleost fish. Gen Comp Endocrinol. 160: 158–167.

Casas E, Keele JW, Shackelford SD, Koohmaraie M, Sonstegard TS, Smith TP, Kappes SM, and Stone RT. 1998. Association of the muscle hypertrophy locus with carcass traits in beef cattle. J Anim Sci. 76: 468–473.

Cash JN, Rejon CA, McPherron AC, Bernard DJ, and Thompson TB. 2009. The structure of myostatin:follistatin 288: insights into receptor utilization and heparin binding. EMBO J. 28: 2662–2676.

Du SJ, Gong Z, Fletcher GL, Shears MA, King MJ, Idler DR, and Hew CL. 1992. Growth enhancement in transgenic Atlantic salmon by the use of an "All Fish" chimeric growth hormone gene construct. Bio/technology. 10: 176–181.

Feng X-H and Derynck R. 2005. Specificity and versatility in TGF-β signaling through SMADs. Ann Rev Cell Dev Biol. 21: 659–693.

Garikipati DK, Gahr SA, and Rodgers BD. 2006. Identification, characterization, and quantitative expression analysis of rainbow trout myostatin-1a and myostatin-1b genes. J Endocrinol. 190: 879–888.

Gross ML, Schneider JF, Moav N, Moav B, Alvarez C, Myster SH, Liu Z, Hallerman EM, Hackett PB, Guise KS, Faras AJ, and Kapuscinski AR. 1992. Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes. Aquaculture. 103: 253–273.

Guo T, Jou W, Chanturiya T, Portas J, Gavrilova O, and McPherron AC. 2009. Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. PLoS ONE. 4: e4937.

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, and Qiu Y. 2002. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem. 277: 40735–40741.

Johnston IA. 2006. Environment and plasticity of myogenesis in teleost fish. J Exp Biol. 209: 2249–2264.

Kambadur R, Sharma M, Smith TPL, and Bass JJ. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 7: 910–915.

Lee C-Y, Hu S-Y, Gong H-Y, Chen MH-C, Lu J-K, and Wu J-L. 2009. Suppression of myostatin with vector-based RNA interference causes a double-muscle effect in transgenic zebrafish. Biochem Biophys Res Commun. 387: 766–771.

Lee SB, Kim YS, Oh M-Y, Jeong I-h, Seong K-B, and Jin H-J. 2010. Improving rainbow trout (*Oncorhynchus mykiss*) growth by treatment with a fish (*Paralichthys olivaceus*) myostatin prodomain expressed in soluble forms in *E. coli*. Aquaculture. 302: 270–278.

Lee S-J. 2004. Regulation of muscle mass by myostatin. Ann Rev Cell Dev Biol. 20: 61–86.

Lee S-J. 2007. Quadrupling muscle mass in mice by targeting TGF-β signaling pathways. PLoS ONE. 2: e789.

Lee S-J and McPherron AC. 2001. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA. 98: 9306–9311.

Lee S-J, Reed LA, Davies MV, Girgenrath S, Goad MEP, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang M-S, Sebald SM, Matzuk M, Li E, Liang L-F, Quattlebaum E, Stotish RL, and Wolfman NM. 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc Natl Acad Sci USA. 102: 18117–18122.

Lin J, Arnold HB, Della-Fera MA, Azain MJ, Hartzell DL, and Baile CA. 2002. Myostatin knock out in mice increases myogenesis and decreases adipogenesis. Biochem Biophys Res Commun. 291: 701–706.

Link BA and Nishi R. 1997. Opposing effects of activin A and follistatin on developing skeletal muscle cells. Exp Cell Res. 233: 350–362.

Liu J-P. 2006. The function of growth/differentiation factor 11 (Gdf11) in rostrocaudal patterning of the developing spinal cord. Development. 133: 2865–2874.

McPherron AC and Lee S-J. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA. 94: 12457–12461.

McPherron AC, Lawler AM, and Lee S-J. 1997. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature. 387: 83–90.

McPherron AC, Huynh TV, and Lee S-J. 2009. Redundancy of myostatin and growth/differentiation factor 11 function. BMC Dev Biol. 9: 1–9.

Medeiros EF, Phelps MP, Fuentes FD, and Bradley TM. 2009. Overexpression of follistatin in trout stimulates increased muscling. Am J Physiol Regul Integr Comp Physiol. 297: R235–R242.

Piper RG, McElwain IB, Orme LE, McCraren JP, Fowler LG, and Leonard JR. 1982. Fish Hatchery Management. US Fish and Wildlife Service, Washington, DC.

Rahman MA, Hwang G-L, Razak SA, Sohm F, and Maclean N. 2000. Copy number related transgene expression and mosaic somatic expression in hemizygous and homozygous transgenic tilapia (*Oreochromis niloticus*). Transgenic Res. 9: 417–427.

Rescan PY. 2005. Muscle growth patterns and regulation during fish ontogeny. Gen Comp Endocrinol. 142: 111–116.

Rodgers BD, Roalson EH, Weber GM, Roberts SB, and Goetz FW. 2007. A proposed nomenclature consensus for the myostatin gene family. Am J Physiol Endocrinol Metab. 292: E371–E372.

Rosenthal N, Kornhauser JM, Donoghue M, Rosen KM, and Merlie JP. 1989. Myosin light chain enhancer activates muscle-specific, developmentally regulated gene expression in transgenic mice. Proc Natl Acad Sci USA. 86: 7780–7784.

Sawatari E, Seki R, Adachi T, Hashimoto H, Uji S, Wakamatsu Y, Nakata T, and Kinoshita M. 2010. Overexpression of the dominant-negative form of myostatin results in doubling of muscle-fiber number in transgenic medaka (*Oryzias latipes*). Comp Biochem Physiol A Mol Integr Physiol. 155: 183–189.

Souza TA, Chen X, Guo Y, Sava P, Zhang J, Hill JJ, Yaworsky PJ, and Qiu Y. 2008. Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Mol Endocrinol. 22: 2689–2702.

Stuart GW, Vielkind JR, McMurray JV, and Westerfield M. 1990. Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. Development. 109: 577–584.

Tsuchida K, Nakatani M, Uezumi A, Murakami T, and Cui X. 2008. Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer. Endocr J. 55: 11–21.

Welt C, Sidis Y, Keutmann H, and Schneyer A. 2002. Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Exp Biol Med. 227: 724–752.

Whitelaw CBA, Springbett AJ, Webster J, and Clark J. 1993. The majority of G_0 transgenic mice are derived from mosaic embryos. Transgenic Res. 2: 29–32.

Whittemore L-A, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan X-Y, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, and Wolfman NM. 2003. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun. 300: 965–971.

Xu C, Wu G, Zohar Y, and Du S-J. 2003. Analysis of myostatin gene structure, expression and function in zebrafish. J Exp Biol. 206: 4067–4079.

Zhu X, Hadhazy M, Wehling M, Tidball JG, and McNally EM. 2000. Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. FEBS Lett. 474: 71–75.

Part 7
Cryopreservation

Chapter 20

Fish Gamete and Embryo Cryopreservation: State of the Art

Paz Herráez, Elsa Cabrita, and Vanesa Robles

BASIC PRINCIPLES OF CELL CRYOPRESERVATION

Cryopreservation is the preservation of life in the frozen state, arresting all the vital functions for indefinite periods of time and allowing the recovery of all the cell, tissue, organ, or organism characteristics after thawing. Metabolic activity is completely prevented at very low temperatures (below −130°C) and only in these conditions can biological samples be preserved indefinitely (Mazur 1984). The possibility of managing "life suspension" is a very useful and interesting tool for science and has been specially applied in reproductive biology. In the context of cattle breeding, cryopreservation of sperm and embryos is a specialized business in which processing tends to be well controlled and quality is accurate. It is considered a key issue in the development of reproductive technologies because it allows the preservation of genetics from rare or valuable breeds (gene banking), provides insurance against death, infertility, or illness, secures genetics for future use, and enables semen and embryos to be marketed or exported.

The adverse effects of freezing are related to the effect of low temperatures on the organization and structure of some organic molecules and, on the other hand, to the physical–chemical processes occurring during freezing and thawing. These processes seriously affect cell structure, function, and their microenvironment, making it a real challenge for cryobiologists to achieve success. During cooling of an aqueous solution, spontaneous ice formation (ice nucleation) only occurs after supercooling well below the freezing point. Different factors, such as the presence of nucleating solutes, increased pressure, or the movement of particles, can facilitate this process. Then, ice crystal growth occurs, and because the solutes are excluded from the ice matrix, they become concentrated in the unfrozen portion of the solution. Finally, when the temperature reaches the eutectic point, the solution becomes solid. Biological materials are mainly aqueous solutions, limited by lipidic membranes and surrounded by aqueous media. During the cooling of cells and tissues, ice nucleation and growth generally occurs within the extracellular space, thus promoting an increase in osmolality and the consequent efflux of water from the cells in order to maintain osmotic equilibrium (Watson and Fuller 2001). Formation of ice crystals inside cells depends on the freezing rate and membrane permeability to water, and can only take place when the freezing rate is fast enough to

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

allow ice nucleation before the cell has been dehydrated. Both cell dehydration and intracellular ice crystal growth are lethal to cells. A similar cell-damaging process also occurs during thawing (fast water flux through the membrane, ice recrystalization, etc.). These damaging outcomes were resolved with the discovery and use of cryoprotectant agents (CPA), such as glycerol (Polge et al. 1949). Further progress in the formulation of cryoprotectant solutions, and the careful design of freezing and thawing regimens to suit the characteristics of the samples to be frozen, have allowed the cryopreservation of many different cell types, some tissues, and other small more complex structures such as corneas and blood vessels.

Some CPAs penetrate the plasma membrane to reach equilibrium between the internal and external medium, while others remain outside the cells. CPAs, such as amines, alcohols, sugars, inorganic salts, and macromolecules, can quantitatively decrease intracellular ice formation by modifying the osmotic pressure, causing freezing point depression and thus reducing the amount of intracellular water available for freezing (Karow 1997). The CPAs can also interact with plasma membrane to help prevent protein denaturation and phase change transitions of phospholipids during freezing. However, CPAs can also have adverse effects due to their toxicity and their effects on osmotic pressure. Dimethylsulfoxide (DMSO) and glycerol, two well-known cryoprotectants, can promote chromosomal abnormalities in oocytes and alter actin polymerization within the cytoskeleton.

Slow, Controlled Freezing

Several protocols using controlled temperature rates have been described in literature. Basically, cells are mixed with a cryoprotectant solution containing a buffered extender of variable composition, one or more permeable CPAs (e.g., DMSO, methanol, glycerol, dimethylacetamide, ethylene glycol) and some external nonpermeable CPAs or other substances that increase the protection of the cell, usually the plasma membrane (e.g., bovine serum albumin, egg yolk components, sugars, soybean proteins, etc.). Once the extender has been incorporated, cells are loaded into specific containers (e.g., straws, macrotubes, or cryovials) and frozen at a controlled freezing rate (usually no faster than -40°C/min). A number of low temperature devices have been designed to perform controlled freezing rates and allow the application of very complex freezing curves (see review from Martínez-Pastor and Adams 2008). Biofreezers can reach temperatures down to -130°C at which point the samples are plunged into liquid nitrogen (N_2l; -196°C) and stored in specially designed containers. In the absence of biofreezers, freezing in N_2l vapors can be substituted as a simple and effective method. Samples (straws, cryovials, etc.) are horizontally arranged in racks over the surface of N_2l inside a Styrofoam box, and the freezing rate is controlled by adjusting the distance of the samples from the N_2l surface. Samples are thawed by submerging them in a temperature-controlled water bath. The thawing temperature depends on the biological material as well as the volume of the frozen samples. In some cases (e.g., embryos), cryoprotectants are removed during or after thawing by immersion in a series of decreasing concentrations of cryoprotectants.

Vitrification

Vitrification is ultrafast freezing performed with a very high concentration of cryoprotectants, during which solidification of the sample is homogeneous without the formation of ice crystals, thus reaching the so-called "glassy solid state." For vitrification, samples are placed in an extender with a very high concentration of CPAs and solutes and plunged directly into liquid nitrogen. Vitrification is a promising and simplifying method especially for complex biological systems such as embryos because the potential injury associated with ice formation can be eliminated (Rall and Fahy 1985).

Cryodamage

It is well known that cryopreservation can damage cells and tissues. It is our goal as cryobiologists to study, reduce, and even avoid some of this damage by improving the protocols and procedures. Cryoinjuries can be the result of (i) the so-called "cold shock" or the effect of low temperatures on the organic molecules and cell structure, (ii) osmotic events taking place during water and CPA flux across the cell membrane, which can expose cells to aggressive osmolalities, modified ion concentrations,

and changes in pH, (iii) toxic effects of CPAs, (iv) ice crystal growth causing mechanical damage, and (v) the formation of free radicals or reactive oxygen species (ROS). The main targets for damage to cells are plasma membranes, which lose their fluidity, integrity, and regionalization; the mitochondria by decreasing their activity; the cytoskeleton, because of interference with polymerization and depolymerization mechanisms; and damage to chromatin that is often fragmented by the freezing and thawing process.

GAMETE CRYOPRESERVATION

Sperm Cryopreservation

Sperm cryopreservation has been one of the most successful areas in cryobiology with applications to human and animal reproductive technologies. The use of frozen sperm is commonplace in cattle production as evidenced by the 2007 export value ($3,690,402) of beef semen from the United States (National Association of Animal Breeders 2008). In addition, the use of cryopreserved sperm from sheep, boar, horse, and domestic carnivores continues to increase.

Sperm cryobanking is also of considerable interest to aquaculture. However, fish sperm differ from those of mammals; therefore, freezing protocols need to be adapted to take their differing characteristics into consideration. Characteristics of fish sperm include immotile spermatozoa in the seminal plasma; activation of motility after dilution in activation solutions (fresh or saltwater in nature); very short duration of motility; short life span; absence of an acrosome (except sturgeons); low numbers of mitochondria; low level of ATP production; and the requirement for a high number of spermatozoa to fertilize each egg (due to the presence of the chorion surrounding the oocyte, which limits the entry of spermatozoa to the micropyle).

Since the first report on crypreservation of herring (*Clupea harengis* L.) sperm in 1953 (Blaxter 1953), a wide number of studies have been carried out on more than fish 200 species (Billard and Zhang 2001; Chao and Liao 2001) with particular emphasis on salmonids, sturgeons, and carp. In the last decade, intensive work has been carried out on several marine species that have a high commercial value, such as turbot (*Scophthalmus maximus*), gilthead seabream (*Sparus aurata*), Atlantic halibut (*Hippoglossus hippoglossus*), and Atlantic cod (*Gadus morhua)*. Detailed cryopreservation protocols for more than 30 fish species developed by different experts have recently been compiled (Cabrita et al. 2008a).

Sperm freezing and thawing protocols should be designed by taking the following steps in to account:

1. Sperm collection. Contamination with faeces, mucus, urine, or other fluids that are able to activate sperm motility must be avoided. Abdominal massage to obtain semen is a common practice, but cannulation or aspiration with syringes (without needles) is often more convenient and there is less danger of contamination. Some species or strains require male sacrifice so that spermatozoa can be obtained directly from the testis (e.g., sex-reversed trout that lack sperm ducts).
2. Sperm quality analysis for selecting suitable samples. It is well known that sperm samples differ in their suitability for freezing. This is because semen quality varies among species, stocks, and even within samples from the same animal where the most suitable quality sperm is available during the middle of the natural reproductive season. It is difficult to evaluate milt quality using a single parameter; thus, the analysis of several indices of cell function and structure is advisable (Rurangwa et al. 2004; Cabrita et al. 2008b). Evaluation of pH, osmolality, and motility (if possible performed with computerized systems that render several motility characteristics), combined with cell viability, can give enough preliminary information for most species.
3. Formulation of the extenders. Extenders are usually buffered saline solutions that were initially designed to imitate seminal plasma composition. The extender must be capable of preventing activation of sperm motility in order to avoid exhaustion during dilution procedures. The presence of K^+ in diluents is required for this purpose in salmonids. It is also essential that the osmolality is no lower than that of the plasma for freshwater species and no higher in saltwater fish. The pH of the extender can vary from 5.2

to 8.5 depending on species, and it is commonly stabilized with tris or phosphate buffers. Many extenders contain sugars such as glucose or sucrose at concentrations of 400–600 mM. One of the most commonly used extenders that was formulated by Mounib in 1978 (sucrose 0.125 mM, $KHCO_3$ 0.1 M and reduced glutathion 0.0065 M in distilled water) is still in use for many species such as rainbow trout (*Oncorhynchus mykiss*), turbot (*S. maximus*), and seabass (*Dicentrarchus labrax)*. With regard to CPAs, most protocols recommend the use of permeable agents such as DMSO, methanol, glycerol, and propylene glycol (PG) at concentrations of 5–15% v/v. The most widely used membrane protectant is egg yolk, while the production of ROS can be controlled by the incorporation of antioxidants such as amino acids (taurine, hypotaurine, lysine), vitamins (ascorbic acid and α-tocoferol succinate), and other compounds (Bucak et al. 2007). The optimal dilution ratio (sperm: extender) varies according to the species: 1:1 is recommended for tilapias, 1:3 for most salmonids, and 1:20 for groupers.

4. Cooling. Controlled cooling is required in mammals to reduce the temperature to approach 0°C. Mammalian sperm dilution is done at 4°C. However, sperm from cold water fish species, and even from some temperate water fishes (e.g., gilthead seabream, *S. aurata*), do not necessarily require controlled cooling protocols.
5. Sperm loading. The simplest freezing method consists of pipetting aliquots of sperm into small holes drilled into dry ice blocks; this is a method that will provide frozen pellets for storage. However, it is safer and more convenient to use cryocontainers such as French straws that were originally developed for bull sperm. These straws (0.25–0.5 cc) provide very good results because of their small diameter, thus providing homogeneous freezing conditions. For species that produce large volumes of sperm, larger containers can be used, such as 5 mL straws or 2 mL cryovials. Freezing of larger volumes of salmonid or cyprinid sperm for routine practices has not yet been achieved.
6. Sperm freezing. Sperm from most fish species are frozen at constant freezing rates ranging from -10 to -40°C per minute and then plunged into liquid nitrogen. This simple freezing method facilitates the use of simple devices and the development of protocols without sophisticated equipment. Nevertheless, the results provided by programmable biofreezers are more accurate and repeatable.
7. Thawing. This should be performed in a water bath under controlled conditions to avoid overheating of the sperm.
8. Fertilization of the eggs. Thawed sperm are very labile and therefore should be used for fertilization immediately after thawing. If water is used to activate motility, their fertilization ability could be reduced. The use of coelomic fluid or specific diluents for fertilization often improves the fertilization results. The sperm to egg ratio should be 10–100 times higher for fertilization with frozen and thawed spermatozoa than would be necessary with nonfrozen sperm. Figure 20.1 illustrates the sperm freezing procedures.

It is believed that sperm from marine fish have a higher quality following freezing than those from freshwater fish. In some species (e.g., turbot, seabream), fertilization rates are similar with fresh or frozen sperm using simple procedures, but in others (e.g., some salmonids and cyprinids), the percentage of fertilized eggs and the hatching rates are reduced. Nevertheless, many aspects of the cryogenic procedures need to be improved to standardize the protocols. Nowadays, there is more concern with the study of specific damage to the cells and how to prevent it. In this regard, evaluation of sperm subpopulations gains importance because sperm is a heterogeneous mixture of different sperm subpopulations rather than a homogeneous sample.

Recently, damage to DNA integrity has been the focus of attention (Labbé et al. 2001; Cabrita et al. 2005; Pérez-Cerezales et al. 2009). DNA injury (fragmentation, oxidization, etc.) could be responsible for malformations in the offspring. Oocyte repair mechanisms are able to correct low levels of damage following fertilization but if damage is high, death before hatch can be expected. Other types of damage could also result in gamete selection by excluding the less cryogenic-resistant spermatozoa from thawed and functional sperm. The use

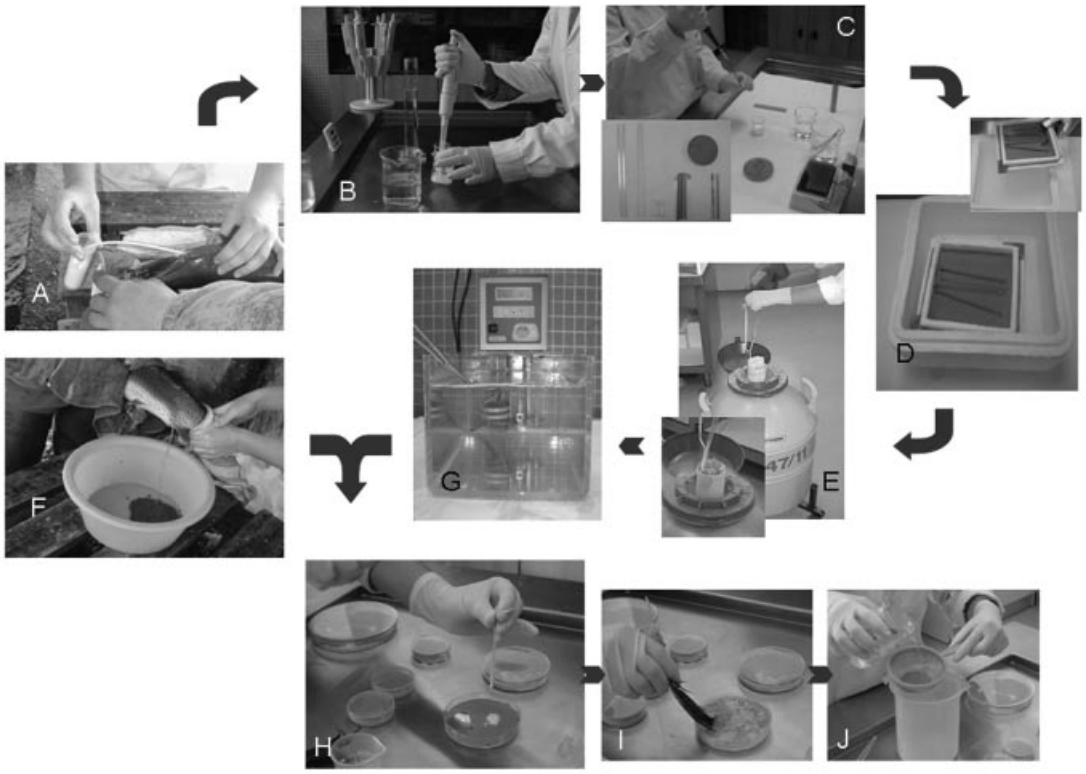


Figure 20.1. Sperm freezing process: (**A**) trout sperm extraction by canulation, (**B**) dilution in a cryoprotectant extender, (**C**) loading in 0.5 cc French straws (insert with different straws, cryovials, and PVA powder for straw sealing), (**D**) freezing over a floating device in a styrofoam box containing N_2l, (**E**) storage in a N_2l tank, (**F**) female stripping, (**G**) sperm thawing in a water bath, and (**H–J**) fertilization. (For color detail, please see the color plate section.)

of crypreserved sperm has been reported to result in high death rates in the progeny of loach (Kopeika et al. 2003). In contrast, Labbé et al. (2001) found that cryopreserved trout sperm had no effect on offspring survival, morphology, and growth. Recently, Martínez-Páramo and coworkers (2009a) used microsatellite analysis to demonstrate that the use of fresh or frozen brown trout (*Salmo trutta*) sperm did not affect the genetic variability of the offspring. Similar results were obtained with African catfish, *Clarias gariepinus* (van der Walt et al. 1993). Comparable studies should be carried out on other species before any generalized conclusions can be made.

The analysis of oxidative stress also deserves more attention. There is still a huge field to explore with regards to improving the protection of cryopreserved cells. Knowledge of basic cell biology and metabolism will help in the design of improved methods for fish sperm cryopreservation.

Oocyte Cryopreservation

Preservation of female gametes is important in order to conserve the maternal genome, including nuclear and mitochondrial DNA as well as the maternal mRNA that is important for early embryonic development. However, fish oocytes represent a much greater challenge for cryobiologists than spermatozoa, and there has been little success to date. As was recently reviewed by Zhang and Lubzens (2008), oocytes have a number of characteristics that make

them very susceptible to cryodamage. During development, oocytes accumulate yolk (lipids, proteins, and in some cases lipid droplets), acquire a large diameter, and develop a number of membrane envelopes that decrease their permeability to water and restrict the diffusion of CPAs. Most of the studies performed in the last decade have tried to preserve oocytes at early developmental stages to minimize these handicaps, and a number of procedures have been developed using isolated ovarian follicles that include immature oocytes and attached cells. At this stage of oocyte development, conditions for cryopreservation appear to be more favorable. If early stage oocytes are cryopreserved, they will need to complete their maturation process in vitro prior to fertilization. Oocyte viability can then be assessed using vital stains, such as trypan blue, or by observation of germinal vesicle breakdown (GVBD).

Most studies on oocyte cryopreservation have been performed with model species such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Research in progress that shows promising results include studies of cryoprotectant toxicity, membrane permeability to cryoprotectants, and chilling sensitivity at different oocyte developmental stages (reviewed in Zhang and Lubzens 2008). In one such study, zebrafish stage III oocytes were frozen using controlled slow cooling with 4 M methanol + 0.2 M glucose in KCl buffer, and more than 85% cells were recorded as viable immediately after thawing as evidenced by trypan blue staining. However, this percentage decreased to 19% after 2 hours of incubation at 22°C (Plachinta et al. 2005). A follow-up study demonstrated that fast thawing and stepwise removal of cryoprotectants improved oocyte survival to 29.5% after 2 hours of incubation (Guan et al. 2008). Unfortunately, the oocytes in these studies eventually became translucent some time after thawing, indicating some kind of damage to the internal compartment, and making the GVBD test to assess progress in development impossible to apply. Taking into account the high chilling sensitivity of these cells, vitrification should also be attempted in future studies. Cryopreservation of oocytes presents a very interesting scientific challenge with many problems to be resolved before we can envisage the possibility of female gamete banking.

EMBRYO CRYOPRESERVATION

Fish embryo cryopreservation (preserving both maternal and paternal genetic information) would be very helpful in the management of reproduction on fish farms. However, it still remains an elusive goal. During recent years, as was reviewed by Robles et al. (2008), researchers have investigated methods to overcome the intrinsic hurdles that this biological model displays against cryopreservation: its large size and multicompartmental structure, the differing osmotic properties of the cells and yolk and the presence of barriers such as the yolk syncytial layer (YSL) that hinder permeability to water and CPAs throughout the embryo (Fig. 20.2; Hagedorn et al. 1997a). The large osmotically inactive water fraction in the embryos contributes to intraembryonic ice formation during freezing and thawing, resulting in cellular damage and, consequently, embryo death. On the other hand, the extreme sensitivity to chilling and cryoprotectant toxicity displayed by early embryonic stages (i.e., exactly those that would theoretically be easier to cryopreserve due to their relatively simple structure) was also another factor hindering success in cryopreservation.

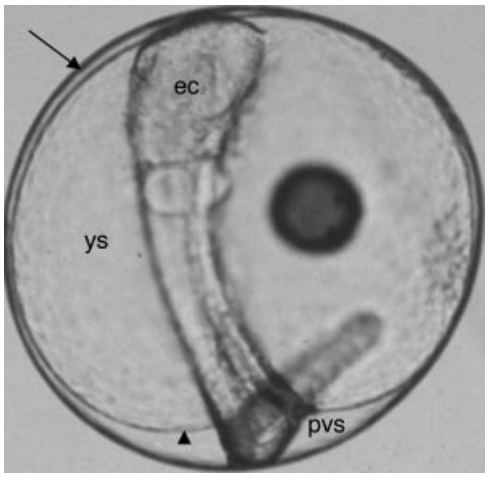


Figure 20.2. Turbot embryo at the tail bud stage showing the different envelopes and compartments: chorion (arrow), yolk syncytial layer (arrowhead), yolk sac (ys), periviteline space (pvs), and embryo compartment (ec). (For color detail, please see the color plate section.)

Chilling sensitivity has been well studied in several species, but zebrafish has been the model most studied. Zhang and Rawson (1995) and Hagedorn et al. (1997b) independently demonstrated that zebrafish embryos at early stages (up to 2 hours postfertilization) were more susceptible to chilling at 0°C than later stages from 50% epiboly to 3-somite. Several approaches have been examined with the aim of reducing chilling injury. For example, the application of anoxia to zebrafish embryos did not result in any improvement (Zhang et al. 2003). On the other hand, Liu et al. (2001) observed significantly reduced chilling sensitivity in zebrafish embryos that were yolk-reduced by microaspiration. Robles et al. (2007) have demonstrated that microinjection of antifreeze proteins (AFPs), specifically AFP type I, into gilthead seabream embryos at the two-cell stage can significantly improve chilling resistance. In contrast, the delivery of high concentrations of other CPAs (DMSO, methanol, ethylene glycol, and sucrose) into the yolk sac of gilthead seabream embryos did not provide significant protection (Beirao et al. 2006).

It is generally assumed that chilling sensitivity, a handicap for slow cooling, could be avoided by using vitrification. However, the required high CPA concentrations inside and outside the embryo could be toxic, and they are also difficult to achieve because of low embryo permeability. Toxicity to cryoprotectants varies among species and with developmental stage and it is usually lower at low temperatures. Several techniques have been used to evaluate the CPA concentration within the embryo and to determine its distribution following treatment with cryoprotectant solutions. Harvey et al. (1983) used a liquid scintillation counter to estimate the permeation of ^{14}C DMSO and ^{3}H glycerol into embryos and found greater permeation into dechorionated embryos than in controls, indicating that the chorion was a barrier to CPAs. Hagedorn et al. (1997a) described very slow water efflux from zebrafish embryos, which decreased in the presence of some CPAs. Their analysis of permeability barriers using magnetic resonance microscopy revealed that methanol is distributed throughout the embryo within 15 minutes of treatment, whereas other cryoprotectants such as DMSO and PG had much lower or no permeation after a 2.5-hour period. They also observed that microinjected DMSO and PG did not diffuse from the yolk to the blastoderm, while they both diffused freely within it. Their data indicated that the YSL was a barrier that blocked the movement of cryoprotectants between the blastoderm and yolk (Hagedorn et al. 1997b). High-performance liquid chromatography has also been employed to measure CPA levels in the embryos of a number of different species including turbot (Cabrita et al. 2003) carp, medaka, and rainbow trout (Suzuki et al. 1995). All of these studies demonstrated low CPA concentrations inside the embryo and emphasized the general difficulty with attaining the high concentrations required for vitrification.

The results of these studies have pushed researchers into a race to find alternative strategies for improving CPA and water penetration into the entire embryo. Janik et al. (2000) directly microinjected CPAs into the yolk of zebrafish embryos to overcome the YSL barrier. However, they found that the postthaw morphology of the YSL was not significantly improved. Hagedorn and her group (Hagedorn et al. 2002) increased YSL permeability by microinjecting zebrafish embryos with mRNA for the aquaporin-3 water channel protein. Their experiments demonstrated that AQP3-GFP (green fluorescent protein) expressing embryos had higher rates of ^{14}C radiolabeled PG uptake than control embryos. Although the blastoderm was expressing AQP3-GFP, its permeability to PG was virtually equal to that of controls; therefore, the additional ^{14}C PG observed in the embryo represented the amount of CPA entering the yolk. This was verified by measuring the ^{14}C PG in the yolk. Their predictive model indicated that the PG concentration for AQP3-GFP embryos could reach 5.4 M, a level that is considered within the range required for vitrification of cells. Their results suggested that AQP3-GFP channels within the YSL allowed CPA penetration into the yolk, thus overcoming this barrier.

Robles and coworkers (2005) considered the winter flounder (*Pseudopleuronectes americanus*) an interesting model to study the effect of vitrification on fish embryos because this subarctic species naturally expressed AFPs (Young and Fletcher 2008; Fletcher and Davies, Chapter 17). AFPs constitute a really efficient method of inhibiting ice crystal formation and preventing the phenomenon of recrystallization, one

of the main problems associated with the thawing of vitrified embryos. Surprisingly, Robles et al. (2005) recorded a small percentage of winter flounder embryos that were able to survive after vitrification. Although none of the embryos hatched in this study, it opened up the possibility of using AFPs for fish embryo cryopreservation. In a later study, Martínez-Páramo et al. (2009b) found that the delivery of AFP I and AFP III into cells of zebrafish embryos by incubation in media containing these proteins significantly reduced damage caused by vitrification in the cellular compartment.

Technological advances, together with the creation of multidisciplinary groups that integrate researchers from different scientific backgrounds, would help to solve the problems inherent with fish embryo cryopreservation. The solution will require increased cryoprotection of the cellular compartment and improved permeabilization and dehydration of the yolk sac. At present, there are two promising areas of research: (i) the use of femtosecond lasers to induce transient pores in zebrafish embryos for delivering exogenous material (Kohli et al. 2007) could be useful for the delivery of CPAs in order to avoid the fragility promoted by microinjection; and (ii) the ectopic expression of aquaporins and AFPs within the embryo could provide lines or strains resistant to the damaging effects of embryo freezing.

BLASTOMERE AND PRIMORDIAL GERM CELL CRYOPRESERVATION

The difficulties that exist with the cryopreservation of fish embryos and oocytes have prompted researchers to explore alternatives for the long-term preservation of maternal and paternal genomes. Two of the most promising alternatives are blastomeres and the primordial germ cells (PGCs). The limiting factor with their use usually appears after cryopreservation with the regeneration of individuals. These cells can be used for cell grafting into receptor embryos where some of them could colonize the gonad.

Rainbow trout blastomeres have been successfully cryopreserved. Calvi and Maisse (1998) provided a method for rainbow trout blastomere cryopreservation using 1,2-propanediol (1.4 M) as the cryoprotectant. Survival rates were as high as 95% for stage 6C cells where they were able to form holoblastic morulas by 24 hours following thaw. The same authors successfully applied this technique to the cryopreservation of carp blastomeres obtaining a survival rate of 96% with the use of late blastula cells (Calvi and Maisse 1999). Kusuda et al. (2002) reported 59.3% survival after the cryopreservation of chum salmon (*Oncorhynchus keta*) blastomeres and Martínez-Páramo et al. (2009b) reported a significant improvement in zebrafish blastomere survival after cryopreservation when type I AFPs were present in the extender. Nevertheless, low frequency of germ line chimerism is usually reported when blastomeres are grafted into the host embryo after thawing (Yamaha et al. 2007).

PGCs have also been successfully cryopreserved (Kobayashi et al. 2007). The importance of the preservation of these cells is clearly presented by Yoshizaki et al. (Chapter 14). The discovery of markers such as vasa (Kbraat et al. 1999) and nanos (Köprunner et al. 2001) was crucial for the visualization and isolation of these cells (Kobayashi et al. 2004). However, single cell isolation is not necessarily required. Kobayashi et al. (2007) successfully cryopreserved genital ridges (the embryonic tissue containing PGCs) from their pvasa-gfp transgenic rainbow trout strain using ethylene glycol (1.8 M) as the CPA. After thawing, the PGCs were transplanted into the peritoneal cavities of nontransgenic trout hatchlings where they differentiated into mature eggs and sperm (identified by GFP expression) within the recipient gonads. Subsequent experiments demonstrated that these previously frozen mature germ cells were fully capable of developing into fertile F1 generation fish.

These advances in fish PGC isolation, cryopreservation, and transplantation open new and promising possibilities, allowing germline chimerism between different species, or in other words, surrogate production. Recently, Fan et al. (2008) has also established PGC cultures from *vasa:RFP* (red fluorescent protein) transgenic zebrafish embryos. Genetic manipulation and selection of these cells in vitro for gene transfer studies is a promising outcome of these studies (see Wong et al., Chapter 12). Cryopreservation provides the best method for the preservation and storage of these new bioengineered lines that are being, and will be, created. These technologies will bring fish model systems (such as zebrafish)

and genetic tools up to the same level as that evident for some mouse strains and undoubtedly will be of considerable value to the aquaculture industry.

APPLICATIONS

The unlimited availability of the abovementioned resources, all of them with a traceable origin and a defined genetic profile, could provide innumerable applications. Cryobanking poses no technical problems with fish sperm from many species, and it is also possible with blastomeres and PGCs. The benefits are related to broodstock management in aquaculture, development of genetic selection programs, marketing of genetics, preservation of biodiversity for conservation programs, or preservation of valuable strains from the biotechnological point of view. The combination of cryobanking with other reproductive technologies (e.g., androgenesis, sex reversal, nuclear transfer, cell grafting) makes the range of applications even wider.

Broodstock Management

The benefits of using sperm cryobanks in commercial farming are more evident in those species in which artificial fertilization is a routine practice, but are also applicable to those species in which natural breeding is the common procedure. Sperm banks (i) avoid the problem of desynchronization of sexual maturity between sexes: sperm can be stored and used when the eggs become available; (ii) allow the programming of mating schemes regardless of the maturation period, breeder availability, or even after male death or gender change in protandric species; (iii) simplify broodstock maintenance: off-season spawning can be induced only in females and cryopreserved sperm can be used to fertilize the eggs; (iv) allow the transport of gametes between fish farms, avoiding the risky and costly animal transport; and (v) provide better sperm economy: it allows the use of the total volume of available milt when the volume of eggs to fertilize is reduced, it is specially valuable when males die or need to be sacrificed for sperm extraction, and facilitates storage when semen is difficult to obtain because of the low volumes that can be stripped from animals in captivity. Moreover, if inbreeding problems are identified, a stock of frozen sperm would undoubtedly be very helpful in the organization of desired mating schemes.

So, why is sperm cryopreservation barely used in commercial fish farming? According to Tiersch (2000), few protocols were adapted and standardized for commercial scale use in the past. Nowadays, efforts to transfer this technology to industry have been fruitful for some species of salmonids, sturgeons, and turbot. The development of international networks for technology transfer or sperm-banking services would be very helpful. There are no feasibility problems in many species and high technology is not required for basic procedures, so it is envisaged that in the near future it will be a more common practice.

Germplasm Banking and Genetic Selection Programs

Cattle production has benefited considerably from the advancements in genetic selection coupled with the use of cryopreserved sperm for artificial insemination during the last decades of the twentieth century. Cryopreserved sperm has allowed worldwide distribution of superior germplasm, by reducing the cost of maintaining genetic lines by continual breeding of animals, and reducing the risk of losing a valuable genetic line through genetic drift, catastrophic accident, impaired reproductive efficiency, or disease outbreaks. In fish, the genetic distance between farmed and wild populations is increasing rapidly due to the advancements in the genetic characterization of cultured and wild stocks that are of value in broodstock selection programs. Sperm cryobanking provides fish farming with the same advantages as any other animal production: crossbreeding programming, storage of germplasm for comparing genetic gains over generations, etc. Sperm cryobanking should thus be used in most farmed fish as a necessary tool for breeding improvement. On the other hand, because the millions of cells present in a single ejaculate represent millions of meiotic recombination events, cryopreserved sperm can be stored for future studies of gene recombination frequency and mapping of genetic loci. Nevertheless, the cryobanking of other sources of germplasm (blastomeres and PGCs), offers a very attractive option for preserving fish populations selected for specific traits (e.g., weight gain, reproductive performance) without losing maternal information, either genomic or

cytoplasmic. However, this technology requires further development before it can be effective.

Conservation of Biodiversity

Germplasm cryobanks are considered the best option for ex situ preservation of biodiversity. A cryobank is a genetic repository, and as such it will not solve population problems. However, a cryobank can guarantee that genes or combination of genes that make any stock unique will not be lost forever. "Frozen populations" serve as genetic stocks to recover the population once the appropriate environmental conditions are restored (Hagedorn and Kleinhans 2000). According to the IUCN (International Union for Conservation of Nature and Natural Resources 2008), 1571 fish species are in danger of becoming extinct, and a higher number of local populations with importance as natural or fishing resources are also in danger because of human activity. Cryobanking requirements for this purpose are different from those of commercial aquaculture ventures; the success of methods for cryobanking and generation of fry is not only related to the number of individuals obtained, but mainly to their representativeness of the original population. Low hatching rates could be good enough for this purpose but the preserved material and recovered seedfish should ideally be genetically unchanged when compared with wild populations.

Sperm freezing of ripe males can even be carried out sometime after death (Routray et al. 2006), and the lack of fertile females for crossbreeding can be solved by the application of interspecific androgenesis. Babiak et al. (2002) reported 0.9% survival of 2-year-old androgenetic trout, and dispermic androgenesis in stellata sturgeon (*Acipenser stellatus*) gave 58% live fry in one month (Grunina et al. 2006). There are few reports on fertility of the resulting androgenotes, which is apparently much reduced in females but little affected in males.

Sperm banks for conservation purposes are in use by several countries, including Finland, the United States, Canada, and Russia; and as far as possible, the origin and genetic background of breeders is carefully controlled.

Developing methods for animal regeneration using cells such as testicular or fin cells would be very useful in this field because mature males are not always available. Nuclear transfer has been assayed with nuclei from medaka somatic cells, and a few fertile adults have been obtained (Bubenshchikova et al. 2005). More progress on oocyte and embryo preservation would also be very useful.

Preservation of Biotechnological Strains

In recent years, advances in biotechnology provide the perfect tool for improving fish aquaculture. Basically, transgenic research, the possibility of chromosome manipulation, and the induction of artificial polyploidy are the main pillars for such advances. All of these genetically modified and chromosome-manipulated strains will be valuable not only for aquaculture but also for biomedical research.

Procedures to create a transgenic fish are becoming more and more diverse and sophisticated in order to increase integration efficiency, reduce mosaicism, and increase transgene transmission to the progeny. Although increased growth, and increased disease and cold resistance are desirable characteristics for farmed species with high economic value, the use of teleosts such as zebrafish as biological models for biomedical research has resulted in the production of many different transgenic lines. In this context, cryopreservation of some of their cells (PGCs, blastomeres, spermatozoa) may provide the optimal method for their preservation.

Keeping in mind the high value of these modified strains, progress with oocyte and embryo cryopreservation is also required; even low survival rates after thawing, in spite of the use of sophisticated freezing procedures, could be considered a big success.

REFERENCES

Babiak I, Dobosz S, Goryczko K, Kuzminski H, Brzuzan P, and Ciesielski S. 2002. Androgenesis in rainbow trout using cryopreserved spermatozoa: the effect of processing and biological factors. Theriogenology. 57: 1229–1249.

Beirao J, Robles V, Herráez MP, Sarasquete C, Dinis MT, and Cabrita E. 2006. Cryoprotectant microinjection toxicity and chilling sensitivity in gilthead seabream (*Sparus aurata*) embryos. Aquaculture. 261(3): 897–903.

Billard B and Zhang T. 2001. Techniques of genetic resource banking in fish. In: Cryobanking the

Genetic Resources: Wildlife Conservation for the Future? (eds. Watson PF and Holt WV). Taylor and Francis Books, London. pp 22–46

Blaxter JHS. 1953. Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature. 172: 1189–1190.

Bubenshchikova E, Ju B, Pristyazhnyuk I, Niwa K, Kaftanovskaya E, Kinoshita M, Ozato K, and Wakamatsu Y. 2005. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. Cloning Stem Cells. 7(4): 255–264.

Bucak MN, Ates S, Ahin A, Varis L, Yüce A, Tekin N, and Akc A. 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen. Microscopic and oxidative stress parameters after freeze–thawing process. Theriogenology. 67: 1060–1067.

Cabrita E, Robles V, Chereguini O, de Paz P, Anel L, and Herráez MP. 2003. Dimethyl sulfoxide influx in turbot embryos exposed to a vitrification protocol. Theriogenology. 60(3): 463–473.

Cabrita E, Robles V, and Herráez MP (eds). 2008a. Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC Press, Taylor and Francis, Boca Raton, FL.

Cabrita E, Robles V, and Herráez MP. 2008b. Sperm quality assessment. In: Methods in Reproductive Aquaculture: Marine and Freshwater Species (eds. Cabrita E, Robles V, and Herraez MP). CRC press, Taylor and Francis, Boca Raton, FL. pp 93–148.

Cabrita E, Robles V, Rebordinos L, Sarasquete C, and Herráez MP. 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and githead sea bream (*Spaurus aurata*) cryopreserved sperm. Cryobiology. 50: 144–153.

Calvi SL and Maisse G. 1998. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres: influence of embryo stage on postthaw survival rate. Cryobiology. 36(4): 255–262.

Calvi SL and Maisse G. 1999. Cryopreservation of carp (*Cyprinus carpio*) blastomeres. Aquat Liv Res. 12: 71–74.

Chao NH and Liao IC. 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture. 197: 161–169.

Fan L, Moon J, Wong T-T, Crodian J, and Collodi P. 2008. Zebrafish primordial germ cell cultures derived from *vasa::RFP* transgenic embryos. Stem Cells Devel. 17: 585–597.

Grunina AS, Recoubratsky AV, Tsvetkova LI, and Barmintsev VA. 2006. Investigation on dispermic androgenesis in sturgeon fish. The first successful production of androgenetic sturgeons with cryopreserved sperm. Int J Refrigeration. 29: 379–386.

Guan M, Rawson DM, and Zhang T. 2008. Cryopreservation of zebrafish (*Danio rerio*) oocytes using improved controlled slow cooling protocols. Cryobiology. 56: 204–208.

Hagedorn M and Kleinhans FW. 2000. Problems and prospects in cryopreservation of fish embryos. In: Cryopreservation in Aquatic Species (eds. Tiersch TR and Mazik PM). World Aquaculture Society, Baton Rouge, LA. pp 161–178.

Hagedorn M, Hsu E, Kleinhans FW, and Wildt DE. 1997a. New approaches for studying the permeability of fish embryos: toward successful cryopreservation. Cryobiology. 34: 335–347.

Hagedorn M, Kleinhans FW, Wildt DE, and Rall WF. 1997b. Chill sensitivity and cryoprotectant permeability of dechorionated zebrafish embryos, *Brachydanio rerio*. Cryobiology. 34(3): 251–263.

Hagedorn M, Lance SL, Fonseca DM, Kleinhans FW, Artimov D, Fleischer R, Hoque ATMS, Hamilton MB, and Pukazhenthi BS. 2002. Altering fish embryos with aquaporin-3: an essential step toward successful cryopreservation. Biol Reprod. 67: 961–966.

Harvey B, Kelley RN, and Ashwood-Smith MJ. 1983. Permeability of intact and dechorionated zebrafish embryos to glycerol and dimethyl sulfoxide. Cryobiology. 20(4): 432–439.

IUCN. 2008. http://www.iucnredlist.org

Janik M, Kleinhans FW, and Hagedorn M. 2000. Overcoming a permeability barrier by microinjecting cryoprotectants into zebrafish embryos (*Brachydanio rerio*). Cryobiology. 41(1): 25–34.

Karow AM. 1997. Pharmacological interventions *in vitro*. In: Reproductive Tissue Banking. Scientific Principles (eds. Karow AM and Critser JK). Academic Press, San Diego, CA. pp 167–229.

Kbraat A, Zandbergen T, Van de Water J, Goos H, and Zivkovic AD. 1999. Characterization of zebrafish primordial germ cells: morphology and early distribution of *vasa* RNA. Dev Dyn. 216: 153–167.

Kobayashi T, Takeuchi Y, Takeuchi T, and Yoshizaki G. 2007. Generation of viable fish from cryopreserved primordial germ cells. Mol Reprod Dev. 74(2): 207–213.

Kobayashi T, Yoshizaki G, Takeuchi Y, and Takeuchi T. 2004. Isolation of highly pure and viable primordial germ cells from rainbow trout by

GFP-dependent flow cytometry. Mol Reprod Dev. 67(1): 91–100.

Kohli V, Robles V, Cancela ML, Acker JP, Waskiewicz AJ, and Elezzabi AY. 2007. An alternative method for delivering exogenous material into developing zebrafish embryos. Biotechnol Bioeng. 98(6): 1230–1241.

Kopeika J, Kopeika E, Zahng T, Rawson DM, and Holt WV. 2003. Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine. Cryobiology. 46: 43–52.

Köprunner MCT, Thisse B, and Raz E. 2001. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev. 15: 2877–2885.

Kusuda S, Teranishi T, and Koide N. 2002. Cryopreservation of chum salmon blastomeres by the straw method. Cryobiology. 45(1): 60–67.

Labbé C, Martoriati A, Devaux A, Maisse G. 2001. Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol Reprod Dev. 60: 397–404.

Liu XH, Zhang T, and Rawson DM. 2001. Effect of cooling rate and partial removal of yolk on the chilling injury in zebrafish embryos. Theriogenology. 55(8): 1719–1731.

Martínez-Pastor F and Adams SL. 2008. Cryobiological material and handling procedures. In: Methods in Reproductive Aquaculture: Marine and Freshwater Species. (eds. Cabrita E, Robles V, and Herraez MP). CRC press, Taylor and Francis, Boca Raton, FL. pp 295–319.

Martínez-Páramo S, Pérez-Cerezales S, Gómez-Romano F, Blanco G, Sánchez JA, and Herráez MP. 2009a. Cryobanking as a tool for conservation of biodiversity: effect of brown trout sperm cryopreservation on the male genetic potential. Theriogenology. 71: 594–604.

Martínez-Páramo S, Barbosa V, Pérez-Cerezales S, Robles V, and Herráez MP. 2009b. Cryoprotective effects of antifreeze proteins delivered into zebrafish embryos. Cryobiology. 58: 128–133.

Mazur P. 1984. Freezing of living cells: mechanisms and implications. Am J Physiol. 247: 125–147.

Mounib MS. 1978. Cryogenic preservation of fish and mammalian spermatozoa. J Reprod Fertil. 53: 13–18.

National Association of Animal Breeders. 2008. www.naab-ss.org

Pérez-Cerezales S, Martínez-Páramo S, Cabrita E, Martínez-Pastor F, Paz P, and Herráez MP. 2009. Evaluation of oxidative DNA damage promoted by storage of sperm from sex-reversed rainbow trout. Theriogenology. 71: 605–613.

Plachinta M, Zahng T, and Rawson DM. 2005. Studies on the effects of certain supplements in cryoprotective medium of zebrafish (*Danio rerio*) oocyte quality after controlled slow cooling. Cryobiology. 51: 405 (abstract).

Polge C, Smith AU, and Parkes AS. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164: 666.

Rall WF and Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature. 313: 573–575.

Robles V, Barbosa V, Herráez MP, Martínez-Páramo S, and Cancela ML. 2007. The antifreeze protein type I (AFP I) increases seabream (*Sparus aurata*) embryos tolerance to low temperature. Theriogenology. 68: 284–289.

Robles V, Cabrita E, Fletcher GL, Shears MA, King MJ, and Herraez MP. 2005. Vitrification assays with embryos from a cold tolerant sub-arctic fish species. Theriogenology. 64(7): 1633–1646.

Robles V, Cabrita E, and Herraez MP. 2008. Cryopreservation of fish embryos: what we know until now? In: Methods in Reproductive Aquaculture: Marine and Freshwater Species (eds. Cabrita E, Robles V, and Herraez MP). CRC Press, Taylor and Francis, Boca Raton, FL. pp 265–294.

Routray P, Choudhary AK, Dash SN, Verma DK, Dash C, Swain P, Jena JK, Gupta SD, and Sarangi N. 2006. Cryopreservation of dead fish spermatozoa several hours after death of Indian major carp, *Labeo rohita* and its successful utilization in fish production. Aquaculture. 261: 1204–1211.

Rurangwa E, Kimeb DE, Olleviera F, and Nasha JP. 2004 The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234: 1–28.

Suzuki T, Komada H, Takai R, Arii K, and Kozima TT. 1995. Relation between toxicity of cryoprotectant DMSO and its concentration in several fish embryos. Fish Sci. 61: 193–197.

Tiersch TR. 2000. Introduction. In: Cryopreservation in Aquatic Species (eds. Tiersch TR and Mazik PM). World aquaculture Society, Baton Rouge, LA. pp xix–xxvi.

Van Der Walt LD, Van Der Bank FH, and Steyn GJ. 1993. The suitability of using cryopreservation of spermatozoa for the conservation of genetic diversity in African catfish (*Clarias gariepinus*). Comp Biochem Physiol A: Physiol. 106: 313–318.

Watson PF and Fuller BJ. 2001. Principles of cryopreservation of gametes and embryos. In: Cryobanking the Genetic Resources. Wildlife Conservation for the Future? (eds. Watson PF and Holt WV). Taylor and Francis Press, London. pp 23–46.

Yamaha E, Saito T, Goto-Kazeto R, and Arai K. 2007. Developmental biotechnology for aquaculture, with special reference to surrogate production in teleost fishes. J Sea Res. 58(1): 8–22.

Young HM and Fletcher GL. 2008. Antifreeze protein gene expression in winter flounder pre-hatch embryos: implications for cryopreservation. Cryobiology. 57: 84–90.

Zhang T, Liu X-H, and Rawson DM. 2003. Effects of methanol and developmental arrest on chilling injury in zebrafish (*Danio rerio*) embryos. Theriogenology. 59(7): 1545–1556.

Zhang T and Lubzens E. 2008. Cryopreservation of fish oocytes. In Methods in Reproductive Aquaculture: Marine and Freshwater Species (eds. Cabrita E, Robles V, and Herraez MP). CRC Press, Taylor and Francis, Boca Raton, FL. pp 251–264.

Zhang T and Rawson DM. 1995. Studies on chilling sensitivity of zebrafish (*Brachydanio rerio*) embryos. Cryobiology. 32(3): 239–246.

Part 8
Environmental Considerations

Chapter 21

The Potential Ecological and Genetic Impacts of Aquaculture Biotechnologies: Eco-Evolutionary Considerations for Managing the Blue Revolution

Darek T.R. Moreau and Ian A. Fleming

INTRODUCTION

Human history has been inextricably shaped by the exploitation of captive animals. In conjunction with plant agriculture, captive-reared animals provided ancestral humans with an abundant and relatively stable food surplus, resulting in increased population densities and stationary communities. The advent of agriculture permitted the diversification of labor, leading to rapid advances in technology and more sophisticated, hierarchical political systems that gradually came to dominate traditional hunter-gatherer societies. Diamond (2002) has suggested that increased human population sizes, a reduction (overexploitation) in large mammal populations, and technological advances leading to food storage were primary reasons for the initial transition to agriculture. A similar, contemporary transition appears to be accompanying the global decline in aquatic food resources, which has been brought about largely by increased human exploitation of aquatic food resources and damage to aquatic ecosystems. In parallel with our Holocene ancestors, we have escalated the development of new technologies for the exploitation of captive aquatic animals (i.e., aquaculture) to supplement capture-based fisheries.

Aquaculture, the cultivation of aquatic organisms, is a primary tool used to supplement depressed global fisheries and, in future, may help conserve heavily exploited populations. Over the last 50 years, the so-called "Blue Revolution" has transformed aquaculture from a localized agricultural activity producing less than 1 million tonnes of food annually to a global industry, producing nearly 60 million tonnes of food annually (FAO 2006; Fig. 21.1). Commercial aquaculture accounts for nearly half of all food fish production worldwide and that proportion is expected to continue to grow.

The rapid growth of aquaculture has been aided tremendously by the application of science. As this book attests, biotechnologies will continue to

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

Figure 21.1. Atlantic salmon sea cage operation in Fortune Bay, Newfoundland, Canada. (For color detail, please see the color plate section.)

provide potential to develop traits advantageous for production. Indeed, biotechnology is partly responsible for the unprecedented growth in volume and diversity of species involved in modern aquaculture production (Duarte et al. 2007). Coming a long way from breeding only the largest and healthiest fish, humans can now select individual fish with the aid of observations at the gene level (e.g., gene expression profiles) or manipulate the genome directly (e.g., transgenesis).

While aquaculture is developing in the biotechnology era, it is also developing in an era of environmental consciousness. There is now considerable pressure to minimize anthropogenic impacts on the environment and biodiversity, which is largely in response to the unprecedented environmental changes we are seeing globally. Commercial aquaculture has not escaped attention, and associated environmental concerns have been a centre of debate (Costa-Pierce 2002; FAO 2006). From an eco-evolutionary perspective, perhaps the most complex concern involves the potential ecological and genetic impacts of aquaculture escapees on surrounding ecosystems.

There are numerous studies on the ecological and genetic effects of aquaculture escapees (reviewed in Utter and Epifanio 2002; Naylor et al. 2005; Thorstad et al. 2008). This chapter, however, focuses on the new environmental challenges introduced by modern biotechnologies. The term biotechnology encompasses a broad collection of tools and applications. Here, we shall restrict our definition to tools applied directly toward the production of aquaculture species with enhanced production traits. Consequently, this chapter concentrates on the similarities and differences between technologies applied in aid of intentional selection (i.e., marker-assisted broodstock development) and those involving direct genetic change (i.e., transgenesis). We shall provide evidence suggesting that the ecological and genetic impacts of transgenic animals may be more difficult to predict than that of animals produced through marker-assisted broodstock development.

GENETIC BACKGROUND

Aquaculture biotechnologies, such as marker-assisted broodstock development, that do not involve direct genetic manipulation remain similar to

traditional intentional selection (i.e., artificial selection based strictly on the phenotypic expression of traits). This is because the only process that differs is that by which parents are selected. In terms of evolutionary processes, they all involve multiple generations of selection that result in concurrent genomic changes and novel phenotypes under polygenic control (Mignon-Grasteau et al. 2005; Jensen 2006). Thus, we will not distinguish between animals produced using such biotechnologies and those produced using traditional artificial selection and will refer to both as farmed strains.

Farmed species tend to have low genetic diversity within and among populations relative to that of wild populations. Genetic similarities appear to be the result of (i) the low number of initial broodstock source populations and (ii) the selection of similar traits over multiple generations. For example, Atlantic salmon (*Salmo salar*) breeding programs have developed with local populations in several places, including Eastern Canada, Norway, and Scotland (Ferguson et al. 2007). For each region, however, programs have either begun by or ended up concentrating on no more than a few strains, which are not likely representative of local population structure (Gjedrem et al. 1991; Gjøen and Bentsen 1997; Glebe 1998). Moreover, some cultured stocks are transplanted or hybridized with local cultured strains, further homogenizing strains used in the industry (Ferguson et al. 2007). Thus, the relatively low number of strains involved in broodstock development contributes to genetic similarities among artificially selected populations and genetic dissimilarities relative to wild populations.

The selective pressures on farmed populations are often very similar; that is, selection for high growth and survival under culture conditions. From an evolutionary perspective, parallel evolution among domesticated strains might be expected, whereby different lineages share genotypic similarities due to similar evolutionary pressures (Foster and Baker 2004; Schluter et al. 2004). Indeed, Roberge et al. (2006, 2008) demonstrated parallels between the transcription profiles of two leading Atlantic salmon aquaculture strains from Eastern Canada and one from Norway. Analogous patterns have also been observed in the transcriptome of closely related cotton congeners (*Gossypium barbadense*, *G. hirsutum*) in response to artificial selection (Chaudhary et al. 2008; Hoavav et al. 2008). Evidence for convergent evolution also exists among *Drosophila subobscura* populations when exposed to laboratory environments over multiple generations (Matos et al. 2000, 2002). These findings suggest that both additive and nonadditive genetic variation may converge among distinct populations experiencing similar pressures from artificial selection.

The phenotypic enhancement strategy of transgenesis is very different from intentional selection. Specific phenotypes are targeted by gene insertion and thus the traditional, polygenic, process of artificial selection can be bypassed. The most common method of creating transgenic fish is currently by cytoplasmic microinjection, where multiple copies of the transgene are inserted into the cytoplasm of a recently fertilized egg and the transgene(s) are incorporated into the developing zygote's genome haphazardly (Du et al. 1992; Iyengar et al. 1996; Twyman 2005). Individuals expressing the desired phenotypic trait are crossed with wild-type fish. The transgenic offspring are then crossed with nontransgenic conspecifics over multiple generations, forming a transgenic line. Successful transgenesis can avoid the time and resources involved in an artificial selection program. Moreover, transgenesis may allow the development of traits not attainable through selective breeding by adding genes that code for proteins unattainable within the host genome (e.g., carbohydrate metabolism or freeze resistance; Fletcher et al. 2004; Fletcher and Davies, Chapter 16). As a result, a transgenic broodstock can be developed in the absence of intentional selection. While transgenesis will more commonly be applied to animal populations that have experienced generations of intentional selection, there may be exceptions. Therefore, some transgenic broodstocks may be more similar genetically (i.e., with exception of the transgene(s)) to their wild source populations than are farmed broodstocks, who have been selected for production traits for generations. This may also reduce the negative fitness consequences for such transgenic animals in the wild. The fitness consequences, however, will depend on how the transgene(s) interacts with the organism's existing genetic architecture, with which it has not coevolved (discussed below).

PHENOTYPIC EXPRESSION

Phenotypic expression among transgenic organisms has been shown to vary by integration position, copy number, construct, strain, and species (Twyman 2005; Gong et al. 2007; Nam et al. 2007). Researchers developing transgenic organisms have noted substantial phenotypic differences between individuals successfully integrating the transgene within the same population. These latter differences are due to epistasis resulting from molecular level variation during the integration process. The genomic location of transgene integration during initial insertion is the major cause of this variation, known as position/integration effects (Iyengar et al. 1996; Twyman 2005). Essentially, epistatic interactions between the transgene and neighboring genes affect the activity of the local molecular region, which may influence the phenotype. Another cause for molecular level variation is the number of transgene copies that integrate into the host genome, known as dosage effects (Twyman 2005). Copy number may affect the amount of protein produced by the transgene loci and, consequently, the overall phenotype. These sources of phenotypic variation are difficult to predict a priori and may have fitness consequences. Continued efforts to develop or adapt more predictable, efficient, and practical gene transfer methods for fish and shellfish species could be an asset for the aquaculture industry and the risk assessment process (Nam et al. 2007).

Evolutionary processes may rearrange transgenes or their location, as with any endogenous gene sequence over time. In some countries, federal legislation requires the demonstration of genetic stability in a transgenic strain for several generations (CEPA 1999; USFDA 2009). Therefore, genetic stability of the transgenic loci and the targeted phenotype will need to be maintained for several generations to commercialize aquaculture biotechnologies (Yaskowiak et al. 2006). However, a few stable generations does not preclude the occurrence of evolutionary processes at the transgene loci in future generations. The effect of genetic recombination, rearrangement, or mutation on the location and structure of the transgene loci cannot be predicted nor can the resulting phenotype or effect on fitness. The likelihood of evolutionary change occurring at the transgene loci may not be any higher than that at other locations in the genome. However, transgenes are invariably designed to behave as genes of major effect; genes that influence the phenotype more so than most genes. Therefore, evolutionary processes at the transgene loci may result in a greater influence on fitness-related traits than would occur at most other loci. Indeed, there is some evidence of transgene instability among some populations of mud loach (*Misgurnus mizolepis*; Nam et al. 1999; Kim et al. 2004). Thus, position effects caused by genetic recombination or rearrangement may change the phenotype of a transgenic line between generations. This is complicated further when we consider the effect of the background genotype on the phenotypic effects induced by the transgene.

The phenotypic response to a stable transgene construct can vary considerably within and between populations and species. Aside from differences caused by construct design, pleiotropy induced by a particular transgene is affected by the composition of, and interaction among, the genes of the receiving animal. The differential response among species to a specified transgene construct is well documented (Nam et al. 2008). There are also examples of this phenomenon among different populations within a species. For example, Devlin et al. (2001) found that the growth response of wild and farmed rainbow trout (*Oncorhynchus mykiss*) populations to transgene (OnMTGH1) introduction differed. The growth response of the wild strain was far greater than that of the farmed strain, which had been selected for rapid growth over several generations. The nontransgenic farm strain, however, outgrew the transgenic wild strain. These results might have been influenced by position or dosage effects. However, a recent study by Neregård et al. (2008a) using growth hormone (GH) implants to compare the growth responses of two wild and one farmed strain of Atlantic salmon (*S. salar*) found similar results (Table 21.2). This confirmed that genetic background can be a key factor in the degree of response to supplementation, whether through transgenesis or implantation.

In summary, intentional selection in aquaculture broodstocks leads to genetic homogeneity brought about by the use of few strains and subsequent parallel evolution. Moreover, intentional selection allows genes and, consequently, phenotypic traits to coevolve over time. Conversely, phenotypes of

different transgenic lines can vary due to transgene position and/or dosage effects or differences in the genetic background of the parent strains. This potential for a higher degree of dissimilarity suggests that the evolutionary pressure on wild populations from interbreeding with transgenic animals may induce a greater array of pleiotropic effects than that observed from interbreeding with farmed animals. However, over time, transgenic strains may experience intentional selection such that fitness reductions are similar to farmed strains. Thus, at least in the absence on intentional selection, the ecological and genetic impacts caused by transgenic animals may be more difficult to predict than that caused by animals produced through traditional intentional selection.

To illustrate the above, we shall compare what is known about potential ecological and genetic effects caused by aquaculture escapees originating from traditional breeding programs with those originating from transgenic manipulation. Despite the diversity of species used in aquaculture, our focus will be on salmonid fishes because of an unfortunate paucity of data addressing ecological and genetic effects of other species. Moreover, salmonids are one of few taxon where fitness-related consequences of transgenesis have been investigated.

DOMESTICATION SELECTION AND DIVERGENCE

Aquaculture animals, including salmon, are genetically and phenotypically distinct from their source wild populations due to the process of domestication selection (e.g., Utter and Epifanio 2002; Ferguson et al. 2007). Domestication selection refers to the different forces affecting genetic change in captive-reared versus wild populations. This genetic change may occur for a number of direct and indirect reasons. Direct genetic change or intentional domestication selection refers to selective breeding for desired traits, such as those targeted in traditional aquaculture practices. Gene transfer biotechnologies are also a direct method of genetic change; however, as previously described, they may differ in fundamental ways.

Domestication selection can also influence both farmed and transgenic animals through indirect genetic change. That is, rearing a population in captivity with no guided selection can lead to divergence from the source population. Unintentional selection may manifest itself through two, most often concurrent, routes. First, changes may result from genetic drift due to an inadvertent sampling bias in the wild flounder population of a captive-bred line, known as a flounder effect (Frankham et al. 2002; Allendorf and Luikart 2007). Second, changes may result from abiotic and biotic differences between wild and captive rearing environments (Price 1999; Einum and Fleming 2001; Huntingford 2004). The result is the potential for indirect selection of traits that increase fitness in the captive environment (Encomio et al. 2005; Shoemaker et al. 2006) or, conversely, relaxed selection on traits that decrease fitness in the wild (Fleming and Gross 1989, 1993; Waples 1999). Unintentional selection is hypothesized to correlate with the number of generations in captivity (Araki et al. 2007; Caroffino et al. 2008). So long as the environment is held constant, farmed and transgenic populations should experience similar unintentional selective pressures; unless there is some sort of unique interaction between the transgene and the environment.

The effects of domestication selection on the genetic and phenotypic characteristics of aquaculture animals lead to various potential environmental impacts upon release into nature. Fig. 21.2 summarizes the mechanisms responsible for such impacts within four categories: direct ecological effects, indirect ecological effects, direct genetic effects, and indirect genetic effects. Most of these potential impacts depend on whether the cultured animals are entering habitat occupied by populations with which they can interbreed. As such, we shall discuss the effects in nonnative (exotic) and native habitats separately.

EFFECTS IN NONNATIVE HABITATS

The potential genetic effects of farmed and transgenic animals are unlikely to differ fundamentally when invading nonnative habitats that lack genetically compatible heterospecific populations. This is because the complex genetic effects associated with species interbreeding and genes introgressing are absent. Interbreeding and introgression are not synonymous. Interbreeding refers only to the act of sexual reproduction between two discrete populations and introgression to the successful transfer of

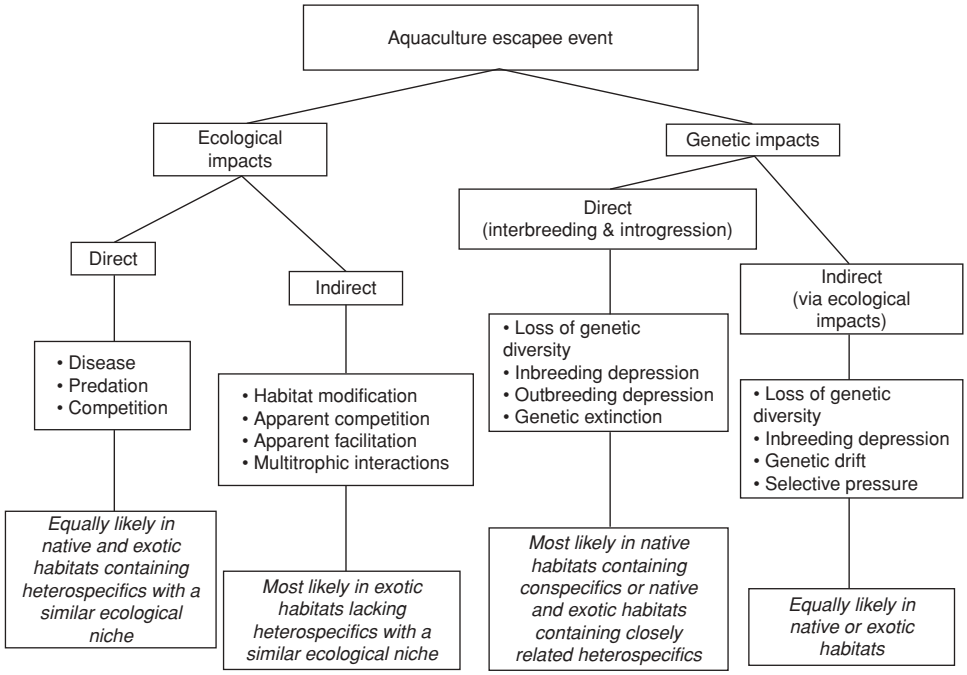


Figure 21.2. Possible environmental impact of aquaculture escapees.

genes from one gene pool to another by interbreeding (Frankham et al. 2002; Allendorf and Luikart 2007). We recognize that exotic habitats may contain closely related species with which an invading population can introgress (reviewed in Allendorf et al. 2001; Allendorf and Luikart 2007); however, hybridization usually occurs at low rates and results in infertile offspring or offspring with greatly reduced reproductive success. Should hybridization result in the introgression of a transgene into a native species, then the effects are likely to become similar to those that occur in native habitats (see below). Here, however, we focus on effects in the absence of introgression.

The effects on ecology and biodiversity resulting from species invasions have been reported for decades (Levine and D'Antonio 1999; Simberloff and von Holle 1999; De Silva et al. 2006). The establishment of an exotic species depends on the frequency and magnitude of the invasion, the relative fitness of the colonizer, and the vulnerability of the ecosystem (Ruesink 2005; Olden et al. 2006). An aquatic species that is farmed extensively is likely to have ample opportunity and sufficient numbers to invade host ecosystems due to the potential for recurring escape events and the scale of farming (Fleming et al. 2000, Bekkevold et al. 2006; Thorstad et al. 2008). Similarly, most aquacultured species are reared in environments where their ability to tolerate local abiotic factors is well understood. Casal (2006) reported a list of the top 18 invasive finfish species reported to have negative effects on local ecosystems, of which 13 (72%) have been used in aquaculture.

Many ecological effects of aquaculture animals are common to both nonnative and native habitats (see below). However, there are also many ecosystem-level abiotic and biotic indirect effects that are more likely in nonnative habitats. Indirect effects refer to changes mediated through a third component (abiotic or biotic) of the ecosystem. Such indirect mechanisms include habitat modification, apparent facilitation, apparent competition, and the trophic cascade effect (Goldschmidt et al. 1993; Shurin et al. 2002; White et al. 2006). Indirect effects are more likely in exotic habitats due to the potential

for exotic species to interact with the environment in a novel manner and, thus, influence community structure and function.

The genetic impacts of aquaculture animals entering nonnative habitats are mediated indirectly through ecological interactions. Indirect genetic effects refer to changes in local heterospecific populations.

Such interactions may change the selective pressures experienced by wild populations, resulting in genotypic and phenotypic adaptations (Waples 1991). In contrast, such changes may manifest themselves through negative interactions, resulting in a reduction in population size and genetic diversity.

EFFECTS WITHIN NATIVE HABITATS

Competition

In terms of risk assessment, the effects of farmed and transgenic animals interacting with wild conspecific or heterospecific populations with which they can interbreed is likely the most critical scenario. This is because the consequences of direct genetic mechanisms, such as an invasion of farmed fish gene complexes or transgenes, are exceptionally difficult to predict (Devlin et al. 2006, 2007; Hindar et al. 2006; Kapuscinski et al. 2007).

Ecological effects can be caused by direct mechanisms, such as disease, predation, and interference competition, or indirect mechanisms, such as exploitative competition (Weir and Grant 2005). In salmonids, competition between farmed and wild conspecifics is well described. Salmonids are interference competitors both as juveniles, when competing for foraging territories, and as adults, when competing for breeding opportunities. Laboratory-based observations generally indicate increased juvenile aggression and poor reproductive behaviors in farmed individuals (Einum and Fleming 2001; Fleming and Petersson 2001; Weir and Grant 2005). These distinct patterns of competition indicate substantial resource overlap; therefore, ecological effects may depend on their respective densities, their relative competitive abilities, and the carrying capacity of the ecosystem (Weber and Faush 2003). However, it should be noted that gene transfer modifications, such as an addition of a gene supporting carbohydrate metabolism (Pitkänen et al. 1999) could affect prey choice and large-scale foraging patterns. There is some evidence of small-scale differences between GH transgenic and nontransgenic coho salmon foraging patterns (Sundström et al. 2007a).

Interbreeding and Introgression

The differences observed in competitive ability suggest differences in survival and reproductive success. Despite lower survival of farmed juveniles, competitive displacement of wild individuals has been observed in Atlantic salmon, indicating potentially negative ecological effects (Fleming et al. 2000; McGinnity et al. 1997, 2003). However, adult farmed strains show poor rates of return to the spawning grounds (McGinnity et al. 2003) and differences in spawning behavior that correlate expectedly with reduced reproductive success (Fleming et al. 1996, 2000; Weir et al. 2004). Reduced reproductive success, however, appears not to carry over to males that mature precociously in fresh water as parr, having never been to the ocean. Males of this alternative life history tactic, which are a fraction of the size of the anadromous males (i.e., males that have been to sea), may breed successfully by sneaking fertilizations (reviewed by Fleming and Reynolds 2004). Farmed males expressing this alternative reproductive phenotype can compete successfully with their wild counterparts for breeding opportunities, leading to equal or even superior reproductive success (Garant et al. 2003; Weir et al. 2005). Thus, the available evidence from salmonid fishes demonstrates that competition can result in the reduced fitness of wild strains. Moreover, farmed individuals have poor lifetime reproductive success in the wild, but can contribute to subsequent generations and may therefore influence the fitness of wild populations (Fleming et al. 2000; McGinnity et al. 2003; Hindar et al. 2006).

The interbreeding of farmed and wild populations gives rise to concerns about the potential negative effects of altering wild gene pools via introgression. Such concerns are based on the evidence suggesting that genetic and phenotypic differentiation between salmonid populations has adaptive significance (Garcia de Leaniz et al. 2007; Carlson and Seamons 2008) and that this would be threatened by introgression.

Captively reared populations usually have lower genetic diversity because they are often closed and

have reduced effective population sizes relative to those in the wild (Frankham 2008). This pattern has been observed repeatedly in aquaculture broodstocks (Exadactylos et al. 1999; Skaala et al. 2005; Frost et al. 2006). Significant one-way gene flow due to escapees could shift the genetic composition of the wild populations toward that of the cultured broodstock (Fleming et al. 2000; McGinnity et al. 2003; Hindar et al. 2006). Subsequent reductions in the genetic diversity of wild populations would make them more vulnerable to environmental change and, in extreme cases, could lead to extinction (Frankham et al. 2002; Allendorf and Luikart 2007; Carlson and Seamons 2008).

Species where there is low gene flow between populations and a high degree of local adaptation, such as salmonids, are particularly vulnerable to outbreeding depression. Outbreeding depression refers to combining alleles from different populations adapted to different environmental conditions, resulting in the reduced fitness of the hybrid population (Wolf et al. 2000; Frankham et al. 2002; Allendorf and Luikart 2007). Further harmful effects may occur if the interbreeding populations disrupt coadapted gene complexes upon recombination in subsequent generations. Coadapted gene complexes are sets of loci that undergo fitness-related epistatic interactions (Wolf et al. 2000; Frankham et al. 2002; Allendorf and Luikart 2007). Consequently, interbreeding between a wild and a captive-reared population may result in outbreeding depression in the hybrid progeny and the breakdown of coadapted gene complexes in subsequent generations. There is fairly consistent empirical evidence of outbreeding depression caused from the interbreeding of wild and farmed or wild and nonlocal salmonid populations (reviewed in Ferguson et al. 2007; Garcia de Leaniz et al. 2007; Fraser 2008).

CASE STUDY OF SALMONID GROWTH ENHANCEMENT

The salmonid growth-enhancement literature consists of studies investigating the fitness-related traits of growth-selected aquaculture (farmed) fish, GH transgenic fish, and fish administered exogenous GH. The latter group has been utilized extensively in the last 15 years as a proxy for transgenic individuals. Typically, this method relies on a continuous, slow-release bovine GH formulation (Posilac®; Monsanto Company; St. Louis, USA) that is implanted into the peritoneal cavity (McLean et al. 1997). The appeal of this substitute is that it allows field comparisons of treated and untreated wild fish, an option not available for transgenic animals. Field experiments allow for the complexity of nature, which cannot be mimicked fully in laboratory environments. Furthermore, the use of wild fish eliminates the potentially confounding effects of the captive rearing environment on phenotypic development. Molecular-level complications associated with the development of transgenic lines, such as position or dosage effects, also need not be a concern.

The obvious limitation of exogenous GH administration is that it is not a complete physiological equivalent of the endogenous GH production induced by a transgene. Scientific understanding of the effects of the GH-insulin-like growth factor I system (GH-IGF-I) on several aspects of salmonid physiology remains uncertain (Björnsson 1997; Björnsson et al. 2002). Moreover, the endocrinological effects of a GH implant compared to a transgene are not known. This may be particularly significant when we consider the inherent complexity of biological cycles. Specifically, the effects of seasonality and age on GH-IGF-I induced phenotypic variation. With this caveat in mind, the effects of exogenous GH administration appear to stimulate similar phenotypic changes as that seen with transgenesis (Tables 21.1 and 21.2). This makes it a useful tool when studying life history periods where we expect that animals are under the influence of sustained GH production, such as that of juvenile salmonids in the spring and summer (Björnsson 1997; Fleming et al. 2002). Therefore, we shall consider exogenous GH administration as analogous to GH transgenesis for the purposes of this chapter and refer to them collectively as GH-enhanced fishes.

Comparing Growth-Selected and GH-Enhanced Phenotypes

A range of similar experiments have been performed on growth selected (farmed) and GH-enhanced (GH transgenic and GH treated) salmonid fishes. Overall, the differences observed for fitness-related traits of farmed and GH-enhanced fishes relative to wild-type

Table 21.1. General Patterns of Fitness-Related Trait Divergence between Wild and Growth Selected (Farmed), Growth Hormone (GH) Transgenic or Growth Hormone-Treated Salmonid Populations Compared in Artificial Laboratory Tanks or Aquaria. Trait Direction Reflects a Comparison of the Growth-Enhanced Relative to Wild-Type Fishes. References Provided Are Not Exhaustive and Are Intended Merely as Examples.

		References		
Phenotypic Trait	Direction	Growth Selected	GH Transgenic	GH Treated
Behavioral				
Dominance/Aggression*	Increased	Einum and Fleming 1997; Fleming and Einum 1997; Metcalfe et al. 2003	Devlin et al. 1999, 2004b	Johnsson and Björnsson 1994; Jönsson et al. 1998; Neregård et al. 2008b
Antipredator behavior	Decreased	Fleming and Einum 1997; Johnsson et al. 2001; Reinhardt 2001; Yamamoto and Reinhardt 2003; Tymchuk et al. 2006	Abrahams and Sutterlin 1999; Sundström et al. 2003, 2007a	Jönsson et al. 1996
Spatial movement	Increased	n/a	Sundström et al. 2007a	Herbert et al. 2001; Jönsson et al. 2003; Johansson et al. 2004, 2005
Feeding motivation/appetite	Increased	Thodesen et al. 1999; Reinhardt 2001; Sanchez et al. 2001; Yamamoto and Reinhardt 2003; Biro et al. 2006	Devlin et al. 1999; Sundström et al. 2003, 2004a; Tymchuk et al. 2005; Lõhmus et al. 2008	Johnsson and Björnsson 1994; Jönsson et al. 1996; Johansson et al. 2005

(*Continued*)

Table 21.1. (*Continued*)

		References		
Phenotypic Trait	Direction	Growth Selected	GH Transgenic	GH Treated
Physiological				
Growth rate	Increased	Gjøen and Bentsen 1997; Glebe 1998; Thodesen et al. 1999; Gjedrem 2000; Fleming et al. 2002; Handeland et al. 2003a, 2003b; Biro et al. 2006; Tymchuk et al. 2006	Du et al. 1992; Cook et al. 2000a, 2000b; Devlin et al. 2001; Leggatt et al. 2003; Sundström et al. 2007b	Johnsson and Björnsson 1994; Neregård et al. 2008a; Sundt-Hansen et al. 2008
Stress response[†]	Decreased	Lepage et al. 2000; Johnsson et al. 2001	n/a	n/a
Metabolic rate	Increased	n/a	Stevens et al. 1998; Cook et al. 2000b, 2000c; Lee et al. 2003; Leggatt et al. 2003; Deitch et al. 2006	Seddiki et al. 1995, 1996
Swimming performance	Decreased	Enders et al. 2004	Farrell et al. 1997; Lee et al. 2003; Deitch et al. 2006	n/a
Hypoxia tolerance	Decreased	n/a	Sundt-Hansen et al. 2007	n/a
Developmental rate	Increased	n/a	Devlin et al. 2004a	n/a

[*]Aggression and dominance are not always increased (Mork et al. 1999; Yamamoto and Reinhardt 2003). See Huntingford (2004) for hypotheses as to why this inconsistency is observed.
[†]Jhingan et al. 2003 found similar stress responses to heat shock in GH transgenic and nontransgenic Coho salmon.

Table 21.2. General Patterns of Fitness-Related Trait Divergence between Wild and Growth Selected (Farmed), Growth Hormone (GH) Transgenic or Growth Hormone-Treated Salmonid Populations Compared in Natural or Near-Natural Laboratory Environments. Trait Direction Reflects a Comparison of the Growth Enhanced Relative to Wild-Type Fishes. Each Numerical Value in the Direction Column Represents One Measurement from a Single Treatment within a Given Study. Thus, Some Studies Contribute Multiple Values to the Direction Column. References Provided Are Not Exhaustive and Are Intended Merely as Examples.

		Direction				
Modification	Trait	>	=	<	References	Comments
GH selected	Survival	3	5	8	Fleming et al. 1996; Einum and Fleming 1997; Fleming and Einum 1997; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003; Biro et al. 2004, 2006; Weir et al. 2004, 2005	Biro et al. 2004, 2006. Observed increased survival when predation was absent or low. Only studies to observe increased survival. Three studies show decreased survival during breeding period.
GH treated	Survival	0	4	1	Johnsson et al. 1999, 2000; Johnsson and Björnsson 2001; Sundt-Hansen et al. 2008	Sundt-Hansen et al. 2008. Decreased survival in mature Atlantic salmon parr during breeding season.
GH transgenic	Survival	0	3	5	Sundström et al. 2004b, 2005	Both studies with first-feeding fry. Demonstrate high susceptibility to predation and low food levels.
GH selected	Growth	8	1	1	Einum and Fleming 1997; Fleming and Einum 1997; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003; Biro et al. 2004, 2006; Tymchuk et al. 2006	

(*Continued*)

Table 21.2. (*Continued*)

Modification	Trait	Direction			References	Comments
		>	=	<		
GH treated	Growth	7	0	1	Johnsson et al. 1996, 1999, 2000; Johnsson and Björnsson 2001; Martin-Smith et al. 2004; Sundt-Hansen et al. 2008, 2009	Martin-Smith et al. 2004. Increased only in summer.
						Sundt-Hansen et al. 2008. Decrease observed is the only study on first-feeding fry.
GH transgenic	Growth	6	8	4	Sundström et al. 2004b, 2005	Sundström et al. 2004b, 2005. Studies with first-feeding fry.
					Sundström et al. 2007b, 2009	Results varied by level of predation, food availability, and rearing environment.
GH selected	Antipredator behavior	0	0	2	Biro et al. 2004, 2006	Inferred.
GH treated	Antipredator behavior	0	0	1	Johnsson et al. 1996	
GH transgenic	Antipredator behavior	0	0	2	Sundström et al. 2004b, 2005	Inferred.
GH transgenic	Predation rate	3	2	0	Sundström et al. 2007b, 2009	
GH treated	Appetite	0	1	0	Johnsson et al. 1996	
GH selected	Competitive behavior	0	2	4	Fleming et al. 1996; Fleming and Einum 1997; Fleming et al. 2000; Weir et al. 2004	Consistent disadvantage for adult breeding males but not females or mature male parr.
GH treated	Competitive behavior	0	2	0	Johnsson et al. 1996; Martin-Smith et al. 2004;	
GH transgenic	Competitive behavior	0	0	1	Bessey et al. 2004	Groups experienced different rearing environments.

GH transgenic	Timing of redd emergence	0	0	1	Sundström et al. 2005	
GH selected	Dispersal/spatial movement	0	0	5	Nagata et al. 1994; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003	Reduced dispersal inferred to be example of competitive displacement.
GH treated	Dispersal/spatial movement	2	3	0	Martin-Smith et al. 2004; Sundt-Hansen et al. 2009	Increased spatial movement but no effect on dispersal or diel movement patterns.
GH transgenic	Dispersal/spatial movement	0	0	2	Sundström et al. 2005, 2007a	
GH treated	Energy reserves	0	3	2	Johnsson et al. 1999, 2000; Neregård et al. 2008a; Sundt-Hansen et al. 2008, 2009	Observed differences most evident in summer and early fall.
GH selected	Reproductive/courtship behavior	1	1	5	Fleming et al. 1996, 2000; Garant et al. 2003; Weir et al. 2004, 2005	Weir et al. 2004. Farmed adult Atlantic salmon males make more spawning attempts but often fail to release sperm.
GH treated	Reproductive/courtship behavior	0	1	0	Sundt-Hansen 2008	Mature Atlantic salmon male parr.
GH transgenic	Reproductive/courtship behavior	0	1	1	Bessey et al. 2004	Divergence found when groups had different rearing environments.
GH selected	Reproductive success	1	1	4	Fleming et al. 1996, 2000; Garant et al. 2003; Weir et al. 2005	Increased and equal reproductive success observed made with mature male Atlantic salmon parr.
GH treated	Reproductive success	0	1	0	Sundt-Hansen 2008	Mature Atlantic salmon male parr.
GH transgenic	Reproductive success	0	1	1	Bessey et al. 2004	Divergence found when groups had different rearing environments.

Figure 21.3. Sibling Atlantic salmon parr produced with one hemizygous growth hormone transgenic parent. These fish were reared in a common artificial environment. Only the upper fish possesses the transgene.

individuals are quite similar in hatchery-type environments (Table 21.1). These data generally indicate increased growth potential and feeding motivation, reductions in antipredator behavior and differences in various physiological correlates relative to wild salmonids (Fig. 21.3).

These similar patterns may correspond to equivalent processes influencing farmed and GH-enhanced animals. A principal phenotypic change resulting from GH transgenesis or exogenous treatment is, unequivocally, increased GH production. The principal phenotype targeted in salmonid aquaculture is growth, and farmed salmonids have been associated with an increase in circulating GH during the juvenile growth phase of their life history (Fleming et al. 2002; Devlin et al. 2009). Thus, similar pluripotent effects on fitness-related traits may be intimately associated with the changes in endocrine growth regulation in both farmed and GH-enhanced salmonids.

Upon comparison of farmed and GH-enhanced individuals relative to wild individuals in more complex environments, variability begins to emerge (Table 21.2). In natural and near-natural experimental streams, evidence of increased growth and decreased antipredator behavior is consistent. However, the direction of fitness-related traits such as survival, reproductive success, energy use, and competitive behavior is inconsistent. Trait divergence between farmed and GH-enhanced individuals relative to wild individuals may reflect genetic and/or environmental differences. For comparison among studies, it is important to identify and, where applicable, control for such sources of trait differentiation. Otherwise, is it difficult to infer whether a genetic predisposition is responsible for trait differences or, alternatively, if differences reflect a plastic response to unique environments. This is not always easy to accomplish because, for example, research cannot be conducted with transgenic organisms in the wild. Therefore, unlike farmed strains, comparing fitness-related traits between wild-reared transgenic and nontransgenic strains may not be possible. In one study, Bessey et al. (2004) showed similar patterns of reproductive trait divergence between GH transgenic coho salmon and wild individuals as has been observed between farmed and wild individuals, suggesting that for these traits, rearing history may be a more critical factor than background genotype (Table 21.2).

When GH-enhanced individuals are compared to wild-type individuals with the same background genotype and rearing history, under natural or near-natural laboratory conditions, many fitness-related traits appear unaffected by treatment (Table 21.2). For example, natural breeding experiments show no differences in courtship behavior and reproductive success between GH-enhanced and -unenhanced individuals when they share the same background genotype and rearing history (Bessey et al. 2004; Sundt-Hansen 2008). Similarly, studies measuring traits in juvenile parr (>2 g) under the threat of predation consistently find increases in growth, but no differences in survival between GH-enhanced and wild individuals (Johnsson et al. 1999; Johnsson et al. 2000; Johnsson and Björnsson 2001; Sundström et al. 2007b; Sundt-Hansen 2008, 2009).

In terms of fitness, many studies on juvenile fish fail to capture a significant period of early life history, the onset of exogenous feeding. The transition to exogenous food resources is a critical period of survival for young fry, where individuals confront an environment saturated with competitors and predators, resulting in high rates of mortality (Elliot 1994; Einum and Fleming 2000; Kennedy et al. 2008). Few studies have measured fitness-related traits in farmed and GH-enhanced fry (<2 g). In near-natural laboratory conditions, Sundström et al. (2004b, 2005)

showed that GH transgenic coho fry experience increased predator-induced morality, reduced dispersal, and equal growth under moderate to high feed levels relative to wild-type fry. However, decreased growth was observed under low feed levels. Conversely, a recent mark-recapture study, where GH-implanted Atlantic salmon fry were released into a natural stream, found no differences in survival or dispersal and reduced growth of GH-implanted relative to control fry (Sundt-Hansen 2008). It is unclear whether these inconsistencies reflect phenotypic differences resulting from intrinsic variation between species, enhancement methods (i.e., transgenesis versus implantation), or experimental environments (e.g., relative levels of predation and competition). It is likely that the inconsistencies are due to a combination of such factors. Nonetheless, controlling for genetic background and rearing environment, differences in fitness-related traits appear less likely between GH-enhanced and -unenhanced individuals compared under near-natural conditions. Furthermore, there is some evidence suggesting that high levels of predation and foraging competition can reduce survival and growth in GH-enhanced individuals at the critical first-feeding stage.

The above discussion highlights a principal reason why transgenic animals may have a lower element of predictability than farmed animals with respect to risk assessment. Farmed individuals have experienced generations of intentional selection for production-relevant traits that are controlled by many genes of small effect. This results in genetic divergence from wild populations. Evidence of consistent, negative fitness consequences have been demonstrated between farmed and wild salmon populations. Because of low strain variation and similar selective pressures among aquaculture broodstocks, variability in the consequences of recombination is principally caused by the response of the receiving population to the breakdown of coadapted polygenic complexes.

In contrast to aquaculture strains, transgenesis can induce growth in fishes of unselected and diverse genetic backgrounds under controlled environments. As genes of major effect, transgenes can have a greater influence on phenotypic expression than most other genes. When growth-enhanced fish are compared to wild-type individuals of similar genetic background, trait differences become evident under laboratory conditions. However, under natural conditions, fitness-related trait differences are variable, weak, or nonexistent, indicative of gene by environment interactions (Devlin et al. 2004b, 2006, 2007; Sundström et al. 2007b). Evidently, it appears that the background genome can moderate the influence of a transgene on the phenotype in response to the environment. This suggests that invading transgenic individuals can experience greater fitness relative to that of invading farmed strains because they have not undergone intentional selection on the whole genome and, therefore, may have a greater capacity to exhibit a plastic response suitable to wild environments. However, transgenic strains that have undergone intentional selection may experience reduced fitness similar to, or greater than, that of farmed strains. The evidence supports this contention, with transgenic strains showing less predictable phenotypic responses to naturalized environments. Consequently, this may also lead to more variable rates of transgene integration into receiving populations. Additionally, the phenotypic effects of transgene position/dosage differences among transgenic lines complicate the ability to accurately conduct risk assessments.

CONCLUSION

Our ability to understand and predict the environmental consequences of transgenic organisms is lagging behind its technological development. We can engineer gene transfer biotechnologies with viable, attractive production traits for aquaculture. However, it is not yet possible to predict the effects of genetic and environmental variation on complex phenotypes, like that formed by interbreeding populations of fish in nature. Transgenesis adds another layer of complexity to the challenge of estimating risks associated with aquaculture escapees. In many countries, regulatory legislation will respond to this scientific uncertainty by administering detailed environmental risk assessments and potential restrictions on the commercialization of gene transfer biotechnologies deemed unsafe.

A long-term solution to dealing with the uncertainty and risk associated with aquaculture biotechnologies is the development of biological containment methods. There is little doubt that aquaculture

biotechnologies will have an increasingly important role in the global food supply of the twenty-first century. The challenge is to ensure that aquaculture development does not contribute to further declines in the health of aquatic ecosystems. Aquaculture biotechnology can play a major role in the sustainable development of the industry by developing practical biological containment methods. The challenge of the biotechnology community is to develop ecologically neutral technologies; that is, animals that cannot interbreed and have little or no impact on the ecosystem. To accomplish this, public and private institutions will need to prioritize research into chromosome-set manipulations, sterility transgenes, sex control, and other such technologies to complement favorable production traits, while recognizing there may also be inherent dangers with these applications as well.

ACKNOWLEDGMENTS

The authors would like to thank Garth Fletcher and Matt Rise for the invitation to contribute to this book. Gratitude is also extended to L. Fredrik Sundström for reviewing an earlier version of this chapter. Funding support for this chapter was provided by a USDA Biotechnology Risk Assessment Grant and an NSERC Discovery grant held by IAF.

REFERENCES

Abrahams MV and Sutterlin A. 1999. The foraging and antipredator behaviour of growth-enhanced transgenic Atlantic salmon. Anim Behav. 58: 933–942.

Allendorf FW and Luikart G. 2007. Conservation and the Genetics of Populations. Blackwell Publishing, Oxford, UK.

Allendorf FW, Leary RF, Spruell P, and Wenburg JK. 2001. The problems with hybrids: setting conservation guidelines. Trends Ecol Evol. 16: 613–622.

Araki H, Cooper B, and Blouin MS. 2007. Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild. Science. 318: 100–103.

Bekkevold D, Hansen MM, and Nielsen EE. 2006. Genetic impact of gadoid culture on wild fish populations: predictions, lessons from salmonids, and possibilities for minimizing adverse effects. J Mar Sci. 63: 198–208.

Bessey C, Devlin RH, Liley NR, and Biagi CA. 2004. Reproductive performance of growth-enhanced transgenic coho salmon. T Am Fish Soc. 133: 1205–1220.

Biro PA, Abrahams MV, Post JR, and Parkinson EA. 2004. Predators select against high growth rates and risk-taking behaviour in domestic trout populations. Proc R Soc Lond B. 271: 2233–2237.

Biro PA, Abrahams MV, Post JR, and Parkinson EA. 2006. Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. J Anim Ecol. 75: 1165–1171.

Björnsson BT. 1997. The biology of salmon growth hormone: from daylight to dominance. Fish Physiol Biochem. 17: 9–24.

Björnsson BT, Johansson V, Benedet S, Einarsdottir IE, Hildahl J, Agustsson T, and Jönsson E. 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiol Biochem. 27: 227–242.

Canadian Environmental Protection Act (CEPA). 1999. New Substances Notification Regulations (Organisms). SOR/2005-248.

Carlson SM and Seamons TR. 2008. A review of fitness and its quantitative genetic components in salmonids: implications for adaptation to future change. Evol Appl. 1: 222–238.

Caroffino DC, Miller M, Kapuscinski AR, and Ostazecki JJ. 2008. Stocking success of local-origin fry and impact of hatchery ancestry: monitoring a new steelhead stocking program in a Minnesota tributary to Lake Superior. Can J Fish Aquat Sci. 65: 309–318.

Casal CMV. 2006. Global documentation of fish introductions: the growing crisis and recommendations for action. Biol Invas. 8: 3–11.

Chaudhary B, Hoavav R, Rapp R, Verma N, Udall JA, and Wendel JF. 2008. Global analysis of gene expression in cotton fibres from wild and domesticated *Gossypium barbadense*. Evol Dev. 10: 567–582.

Cook JT, McNiven MA, Richardson GF, and Sutterlin AM. 2000a. Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture. 188: 15–32.

Cook JT, McNiven MA, and Sutterlin AM. 2000b. Metabolic rate of pre-smolt growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture. 188: 33–45.

Cook JT, Sutterlin AM, and McNiven MA. 2000c. Effect of food deprivation on oxygen consumption and body composition of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture. 188: 47–63.

Costa-Pierce BA. 2002. Ecological Aquaculture: The Evolution of the Blue Revolution. Blackwell Science, Malden.

De Silva SS, Nguyen TTT, Abery NWA, and Amarasinghe US. 2006. An evaluation of the role and impacts of alien finfish in Asian inland aquaculture. Aquacult Res. 37: 1–17.

Deitch EJ, Fletcher GL, Petersen LH, Costa IASF, Shears MA, Driedzic WR, and Gamperl AK. 2006. Cardiorespiratory modifications, and limitations, in post-smolt growth hormone transgenic Atlantic salmon *Salmo salar*. J Exp Biol. 209: 1310–1325.

Devlin RH, Johnsson JI, Smailus DE, Biagi CA, Jonsson E, and Bjornsson BTh. 1999. Increased ability to compete for food by growth hormone-transgenic coho salmon *Oncorhynchus kisutch* (Walbaum). Aqua Res. 30: 479–482.

Devlin RH, Biagi CA, Yesaki TY, Smailus DE, and Byatt JC. 2001. Growth of domesticated transgenic fish: a growth-hormone transgene boosts the size of wild but not domesticated trout. Nature. 409: 781–782.

Devlin RH, Biagi CA, and Yesaki TY. 2004a. Growth, viability and genetic characteristics of GH transgenic coho salmon strains. Aquaculture. 236: 607–632.

Devlin RH, D'Andrade M, Uh M, and Biagi CA. 2004b. Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. Proc Nat Acad Sci USA. 101: 9303–9308.

Devlin RH, Sakhrani D, Tymchuk WE, Rise ML, and Goh B. 2009. Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (*Oncorhynchus kisutch*). Proc Nat Acad Sci USA. 106: 3047–3052.

Devlin RH, Sundström LF, Johnsson JI, Fleming IA, Hayes KR, Ojwang WO, Bambaradeniya C, and Zakaraia-Ismail M. 2007. Assessing ecological effects of transgenic fish prior to entry into nature. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds. Kapuscinski AR, Hayes KR, Li S, and Dana G). Vol. 3. CAB International, Cambridge. pp 151–187.

Devlin RH, Sundström F, and Muir WM. 2006. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends Biotech. 24: 89–98.

Diamond J. 2002. Evolution, consequences and future of plant and animal domestication. Nature. 418: 700–709.

Du SJ, Gong Z, Fletcher GL, Shears MA, King MJ, Idler DR, and Hew CL. 1992. Growth enhancement in transgenic atlantic salmon by the use of an "all-fish" chimeric growth hormone gene construct. Biotechology. 10: 179–187.

Duarte CM, Marbá N, and Holmer M. 2007. Rapid domestication of marine species. Science. 316: 382–383.

Einum S and Fleming IA. 1997. Genetic divergence and interactions in the wild among native, farmed and hybrid Atlantic salmon. J Fish Biol. 50: 634–651.

Einum S and Fleming IA. 2000. Selection against late emergence and small offspring in Atlantic salmon (*Salmo salar*). Evolution. 54: 628–639.

Einum S and Fleming IA. 2001. Implications of stocking: ecological interactions between wild and released salmonids. Nord J Freshwat Res. 75: 56–70.

Elliot JM. 1994. Quantitative Ecology and the Brown Trout. Oxford University Press, Oxford.

Encomio VG, Stickler SM, Allen SK, and Chu FL. 2005. Performance of "natural dermo-resistant" oyster stocks—survival, disease, growth, condition and energy reserves. J Shell Res. 24: 143–155.

Enders E, Boisclair D, and Roy AG. 2004. The costs of habitat utilization of wild, farmed, and domesticated juvenile Atlantic salmon (*Salmo salar*). Can J Aquat Sci. 61: 2302–2313.

Exadactylos A, Greffen AJ, and Thorpe JP. 1999. Growth and genetic variation in hatchery-reared larval and juvenile Dover sole, *Solea solea* (L.). Aquaculture. 176: 209–226.

FAO (Food and Agriculture Organization). Inland Water Resources and Aquaculture Service, Fisheries Resources Division. 2006. State of world aquaculture. FAO Fisheries Technical Paper 500. FAO Information Division, Rome.

Farrell AP, Bennett W, and Devlin RH. 1997. Growth-enhanced transgenic salmon can be inferior swimmers. Can J Zool. 75: 335–337.

Ferguson A, Fleming IA, Hindar K, Skaala Ø, McGinnity P, Cross T, and Prodöhl P. 2007. Farm escapes. In: Atlantic Salmon: Genetics, Conservation and Management (ed. Verspoor E, Stradmeyer L, and Nielsen J). Blackwell Publishing, Oxford. pp 367–409.

Fleming IA, Agustsson T, Finstad B, Johnsson JI, and Björnsson BT. 2002. Effects of domestication on growth physiology and endocrinology of Atlantic salmon (*Salmo salar*). Can J Fish Aquat Sci. 59: 1323–1330.

Fleming IA and Einum S. 1997. Experimental tests of genetic divergence of farmed from wild Atlantic salmon due to domestication. ICES J Mar Sci. 54: 1051–1063.

Fleming IA and Gross MR. 1989. Evolution of adult female life-history and morphology in a pacific salmon (Coho, *Oncorhynchus kisutch*). Evolution. 43: 141–157.

Fleming IA and Gross MR. 1993. Breeding success of hatchery and wild coho salmon (*Oncorhynchus kisutch*) in competition. Ecol App. 3: 230–245.

Fleming IA, Hindar K, Mjølnerod IB, Jonsson B, Balstad T, and Lamberg A. 2000. Lifetime success and interactions of farm salmon invading a native population. Proc R Soc Lond B: 267: 1517–1523.

Fleming IA, Jonsson B, Gross MR, and Lamberg A. 1996. An experimental study of the reproductive behaviour and success of farmed and wild Atlantic salmon (*Salmo salar*). J App Ecol. 33: 893–905.

Fleming IA and Petersson E. 2001. The ability of released, hatchery salmonids to breed and contribute to the natural productivity of wild populations. Nord J Freshwat Res. 75: 71–98.

Fleming IA and Reynolds JD. 2004. Salmonid breeding systems. In: Evolution Illuminated, Salmon and Their Relatives (eds. Hendry AP and Stearns SC), Oxford University Press, Oxford. pp 264–294.

Fletcher GL, Shears MA, Yaskowiak ES, King MJ, and Goddard SV. 2004. Gene transfer: potential to enhance the genome of atlantic salmon for aquaculture. Aust J Exp Agr. 44: 1095–1100.

Foster SA and Baker JA. 2004. Evolution in parallel: new insights from a classic system. Trends Ecol Evol. 19: 456–459.

Frankham R. 2008. Genetic adaptation to captivity. Mol Ecol. 17: 325–333.

Frankham R, Ballou JD, and Briscoe DA. 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge.

Fraser DJ. 2008. How well can captive breeding programs conserve biodiversity? A review of salmonids. Evol Appl. 1: 535–586.

Frost LA, Evans BS, and Jerry DR. 2006. Loss of genetic diversity due to hatchery culture practices in barramundi (*Lates calcarifer*). Aquaculture. 261: 1056–1064.

Garant D, Fleming IA, Einum S, and Bernatchez L. 2003. Alternative male life-history tactics as potential vehicles for speeding introgression of farm salmon traits into wild populations. Ecol Lett. 6: 541–549.

Garcia de Leaniz C, Fleming IA, Einum S, Verspoor E, Jordan WC, Consuegra S, Aubin-Horth N, Lajus D, Letcher BH, Youngson AF, Webb J, Vøllestad LA, Villanueva B, Ferguson A, and Quinn TP. 2007. A critical review of inherited adaptive variation in Atlantic salmon. Biol Rev. 82: 173–211.

Gjedrem T. 2000. Genetic improvement of cold-water fish species. Aqua Res. 31: 541–549.

Gjedrem T, Gjøen HM, and Gjerde B. 1991. Genetic origin of Norwegian farmed salmon. Aquaculture. 98: 41–50.

Gjøen HM and Bentsen HB. 1997. Past, present, and future of genetic improvement in salmon aquaculture. ICES J Mar Sci. 54: 1009–1014.

Glebe BD. 1998. East coast salmon aquaculture breeding programmes: history and future. Department of Fisheries and Oceans Canadian Stock Assessment 98/157, Ottawa.

Goldschmidt T, Witte F, and Wanink J. 1993. Cascading effects of the introduced Nile perch on the detritivorous/phytoplanktivorous species in the sublittoral areas of Lake Victoria. Conserv Biol. 7: 686–700.

Gong Z, Maclean N, Devlin RH, Martinez R, Omitogun O, and Estrada MP. 2007. Gene construct and expression: information relevant for risk assessment and management. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds. Kapuscinski AR, Hayes KR, Li S, and Dana G). Vol. 3. CAB International, Cambridge. pp 61–94.

Handeland SO, Bjornsson BT, Arnesen AM, and Stefansson SO. 2003b. Seawater adaptation and growth of post-smolt Atlantic salmon (*Salmo salar*) of wild and farmed strains. Aquaculture. 220: 367–384.

Handeland SO, Porter M, Bjornsson BT, and Stefansson SO. 2003a. Osmoregulation and growth in a wild and a selected strain of Atlantic salmon smolts on two photoperiod regimes. Aquaculture. 222: 29–43.

Herbert NA, Armstrong JD, and Björnsson BT. 2001. Evidence that growth hormone-induced elevation in routine metabolism of juvenile Atlantic salmon is a result of increased spontaneous activity. J Fish Biol. 59: 754–757.

Hindar K, Fleming IA, McGinnity P, and Diserud O. 2006. Genetic and ecological effects of salmon farming on wild salmon: modeling from experimental results. ICES J Mar Sci. 63: 1234–1247.

Hoavav R, Chaudhary B, Udall JA, Flagel L, and Wendel JF. 2008. Parallel domestication, convergent evolution and duplicated gene recruitment in allopolyploid cotton. Genetics. 179: 1725–1733.

Huntingford FA. 2004. Implications of domestication and rearing conditions for the behaviour of cultivated fishes. J Fish Biol. 65: 122–142.

Iyengar A, Müller F, and McLean N. 1996. Regulation and expression of transgenes in fish: a review. Transgenic Res. 5: 147–166.

Jensen P. 2006. Domestication from behaviour to genes and back again. App Anim Behav Sci. 97: 3–15.

Jhingan E, Devlin RH, and Iwama GK. 2003. Disease resistance, stress response and effects of triploidy in growth hormone transgenic coho salmon. J Fish Biol. 63: 806–823.

Johansson V, Winberg S, and Björnsson BT. 2005. Growth hormone-induced stimulation of swimming and feeding behaviour of rainbow trout is abolished by the D-1 dopamine antagonist SCH23390. Gen Comp Endocr. 141: 58–65.

Johansson V, Winberg S, Jönsson E, Hall D, and Björnsson BT. 2004. Peripherally administered growth hormone increases brain dopaminergic activity and swimming in rainbow trout. Horm Behav. 46: 436–443.

Johnsson JI and Björnsson BTh. 1994. Growth hormone increases growth rate, appetite and dominance in juvenile rainbow trout, *Oncorhynchus mykiss*. Anim Behav. 48: 177–186.

Johnsson JI and Björnsson BTh. 2001. Growth-enhanced fish can be competitive in the wild. Funct Ecol. 15: 654–659.

Johnsson JI, Höjesjö J, and Fleming IA. 2001. Behavioural and heart rate responses to predation risk in wild and domesticated Atlantic salmon. Can J Fish Aquat Sci. 58: 788–794.

Johnsson JI, Jönsson E, Petersson E, Jarvi T, and Bjornsson BT. 2000. Fitness-related effects of growth investment in brown trout under field and hatchery conditions. J Fish Biol. 57: 326–336.

Johnsson JI, Petersson E, Jönsson E, Björnsson BT, and Jarvi T. 1996. Domestication and growth hormone alter antipredator behaviour and growth patterns in juvenile brown trout, *Salmo trutta*. Can J Fish Aquat Sci. 53: 1546–1554.

Johnsson JI, Petersson E, Jönsson E, and Björnsson BT. 1999. Growth hormone-induced effects on mortality, energy status and growth: a field study on brown trout (*Salmo trutta*). Funct Ecol. 13: 514–522.

Jönsson E, Johnsson JI, and Björnsson BT. 1996. Growth hormone increases predation exposure of rainbow trout. Proc R Soc Lond [Biol]. 263: 647–651.

Jönsson E, Johnsson JI, and Björnsson BT. 1998. Growth hormone increases aggressive behavior in juvenile rainbow trout. Horm Behav. 33: 9–15.

Jönsson E, Johansson V, Björnsson BTh, and Winberg S. 2003. Central nervous system actions of growth hormone on brain monoamine levels and behaviour of juvenile rainbow trout. Horm Behav. 43: 367–374.

Kapuscinski AR, Hard JJ, Paulson KM, Neira R, Ponniah A, Kamonrat W, Mwanja W, Fleming IA, Gallardo J, Devlin RH, and Trisak J. 2007. Approaches to assessing gene flow. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish. (eds. Kapuscinski AR, Hayes KR, Li S, and Dana G). Vol. 3. CAB International, Cambridge. pp 112–150.

Kennedy BP, Nislow KH, and Folt C. 2008. Habitat-mediated foraging limitations drive survival bottlenecks for juvenile salmon. Ecology. 89: 2529–2541.

Kim DS, Kim BS, Lee SJ, Park IS, and Nam YK. 2004. Comparative analysis of inherited patterns if the transgene in transgenic mud loach (*Misgurnus mizolepis*) lines carrying the CAT reporter gene. Fish Sci. 70: 201–210.

Lee CG, Farrell AP, Lotto A, Hinch SG, and Healey MC. 2003. Excess post-exercise oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon following critical speed swimming. J Exp Biol. 206 (18): 3253–3260.

Leggatt RA, Devlin RH, Farrell AP, and Randall DJ. 2003. Oxygen uptake of growth hormone transgenic coho salmon during starvation and feeding. J Fish Biol. 62: 1053–1066.

Lepage O, Øverli Ø, Petersson E, Järvi T, and Winberg S. 2000. Differential stress coping in wild and domesticated sea trout. Brain Behav Evol. 56: 259–268.

Levine JM and D'Antonio CM. 1999. Elton revisited: a review of evidence linking diversity and invasibility. Oikos 87: 15–26.

Lõhmus M, Raven PA, Sundström LF, and Devlin RH. 2008. Disruption of seasonality in growth hormone-transgenic coho salmon (*Oncorhynchus kisutch*) and the role of cholecystokinin in seasonal feeding behavior. Horm Behav. 54: 506–513.

Martin-Smith KM, Armstrong JD, Johnsson JI, and Bjornsson BT. 2004. Growth hormone increases growth and dominance of wild juvenile Atlantic salmon without affecting space use. J Fish Biol. 65: 156–172.

Matos M, Rose MR, Pite MTR, Rego C, and Avelar T. 2000. Adaptation to the laboratory environment in *Drosophila subobscura*. J Evol Biol. 13: 9–19.

McGinnity P, Stone C, Taggart JB, Cooke D, Cotter D, Hynes R, McCamley C, Cross T, and Ferguson A. 1997. Genetic impact of escaped farmed Atlantic salmon (*Salmo salar* L.) on native populations: use of DNA profiling to assess freshwater performance

of wild, farmed, and hybrid progeny in a natural river environment. ICES J Mar Sci. 54: 998–1008.

McGinnity P, Prodohl P, Ferguson A, Hynes R, Maoileidigh NO, Baker N, Cotter D, O'Hea B, Cooke D, Rogan G, Taggart J, and Cross T. 2003. Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farm salmon. Proc R Soc Lond B. 270: 2443–2450.

McLean E, Devlin, RH, Byatt JC, Clarke WC, and Donaldson EM. 1997. Impact of a controlled release formulation of recombinant bovine growth hormone upon growth and seawater adaptation in coho (*Oncorhynchus kisutch*) and Chinook (*Oncorhynchus tshawytscha*) salmon. Aquaculture. 156: 113–128.

Metcalfe NB, Valdimarsson SK, and Morgan IJ. 2003. The relative roles of domestication, rearing environment, prior residence and body size in deciding territorial contests between hatchery and wild juvenile salmon. J App Ecol. 40: 535–544.

Mignon-Grasteau S, Boissy A, Bouix J, Faure JM, Fisher AD, Hinch GN, Jensen P, Le Neindre P, Mormede P, Prunet P, Vandeputte M, and Beaumont C. 2005. Genetics of adaptation and domestication in livestock. Lab Anim Sci. 93: 3–15.

Mork OI, Bjerkeng B, and Rye M. 1999. Aggressive interactions in pure and mixed groups of juvenile farmed and hatchery-reared wild Atlantic salmon *Salmo salar* L. in relation to tank substrate. Aqua Res. 30: 571–578.

Nagata M, Nakajima M, and Fujiwara M. 1994. Dispersal of wild and domestic masu salmon fry (*Oncorhynchus-masou*) in an artificial channel. J Fish Biol. 45: 99–109.

Nam YK, Noh CH, and Kim DS. 1999. Transmission and expression of an integrated reporter construct in three generations of transgenic mud loach (*Misgurnus mizolepis*). Aquaculture. 172: 229–245.

Nam YK, Maclean N, Fu C, Pandian TJ, and Eguia MRR. 2007. Development of transgenic fish: scientific background. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds. Kapuscinski AR, Hayes KR, Li S, and Dana G). Vol. 3. CAB International, Cambridge. pp 61–94.

Nam YK, MacLean N, Hwang G, and Kim DS. 2008. Autotransgenic and allotransgenic manipulation of growth traits in fish for aquaculture: a review. J Fish Biol. 72: 1–26.

Naylor R, Hindar K, Fleming IA, Goldburg R, Williams S, Volpe J, Whoriskey F, Eagle J, Kelso D, and Mangel M. 2005. Fugitive salmon: assessing the risks of escaped fish from net-pen aquaculture. Bioscience. 55: 427–437.

Neregård L, Sundt-Hansen L, Hindar K, Einum S, Johnsson JI, Devlin RH, Fleming IA, and Björnsson BTh. 2008a. Wild Atlantic salmon *Salmo salar* L. strains have greater growth potential than a domesticated strain selected for fast growth. J Fish Biol. 73: 79–95.

Neregård L, Sundt-Hansen L, Björnsson BTh, and Johnsson JI. 2008b. Growth hormone affects behaviour of wild brown trout Salmo trutta in territorial owner—intruder conflicts. J Fish Biol. 73: 2341–2351.

Olden JD, Poff NL, and Bestgen KR. 2006. Life-history strategies predict fish invasions and extirpations in the Colorado River Basin. Ecol Mono. 76: 25–40.

Pitkänen TI, Krasnov A, Reinisalo M, and Mölsä H. 1999. Transfer and expression of glucose transporter and hexokinase genes in salmonid fish. Aquaculture. 173: 319–332.

Price EO. 1999. Behavioral development in animals undergoing domestication. App Anim Behav Sci. 65: 245–271.

Reinhardt UG. 2001. Selection for surface feeding in farmed and sea-ranched masu salmon juveniles. Trans Am Fish Soc. 130: 155–158.

Roberge C, Einum S, Guderley H, and Bernatchez L. 2006. Rapid parallel evolutionary changes of gene transcription profiles in farmed Atlantic salmon. Mol Ecol. 15: 9–20.

Roberge C, Normandeau E, Einum S, Guderley H, and Bernatchez L. 2008. Genetic consequences of interbreeding between farmed and wild Atlantic salmon: insights from the transcriptome. Mol Ecol. 17: 314–324.

Ruesink JL. 2005. Global analysis of factors affecting the outcome of freshwater fish introductions. Conserv Biol. 19: 1883–1893.

Sanchez MP, Chevassus B, Labbé L, Quillet E, and Mambrini M. 2001. Selection for growth of brown trout (*Salmo trutta*) affects feed intake but not feed efficiency. Aquat Living Resour. 14: 41–48.

Schluter D, Clifford EA, Nemethy M, and McKinnon JS. 2004. Parallel evolution and inheritance of quantitative traits. Amer Nat. 163: 809–822.

Seddiki H, Boeuf G, Maxime V, and Peyraud C. 1996. Effects of growth hormone treatment on oxygen consumption and sea water adaptability in Atlantic salmon parr and pre-smolts Aquaculture. 148: 49–62.

Seddiki H, Maxime V, Boeuf G, and Peyraud C. 1995. Effects of growth-hormone on plasma ionic

regulation, respiration and extracellular acid-base status in trout (*Oncorhynchus mykiss*) transferred to seawater. Fish Physiol Biochem. 14: 279–288.

Shoemaker CA, Lim C, Yildirim-Aksoy M, Welker TL, and Klesius PH. 2006. Growth response and acquired resistance of Nile tilapia, *Oreochromis niloticus* (L.) that survived *Streptococcus iniae* infection. Aquacult Res. 37: 1238–1245.

Shurin JB, Borer ET, Seabloom EW, Anderson K, Blanchette CA, Briotman B, Cooper SD, and Halpern BS. 2002. A cross-ecosystem comparision of the strength of trophic cascades. Ecol Let. 5: 785–791.

Simberloff D and Von Holle B. 1999. Positive interactions of nonindigenous species: invasional meltdown? Biol Invas. 1: 21–32.

Skaala Ø, Taggart JB, and Gunnes K. 2005. Genetic differences between five major domesticated strains of Atlantic salmon and wild salmon. J Fish Biol. 67: 118–128.

Stevens DE, Sutterlin A, and Cook T. 1998. Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. Can J Fish Aquat Sci. 55: 2028–2035.

Sundström FL, Devlin RH, Johnsson JI, and Biagi CA. 2003. Vertical position reflects increased feeding motivation in growth hormone transgenic coho salmon (*Oncorhynchus kisutch*). Ethology. 109: 701–712.

Sundström FL, Lohmus M, Johnsson JI, and Devlin RH. 2004b. Growth hormone transgenic salmon pay for growth potential with increased predation mortality. Proc R Soc Lond B. 271 (Suppl. 5): S350–S352.

Sundström FL, Lohmus M, Devlin RH, Johnsson JI, Biagi CA, and Bohlin T. 2004a. Feeding on profitable and unprofitable prey: comparing the behaviour of growth-enhanced transgenic and normal coho salmon. Ethology. 110: 381–396.

Sundström LF, Lõhmus M, and Devlin RH. 2005. Selection on increased intrinsic growth rates in coho salmon, *Oncorhynchus kisutch*. Evolution. 59: 1560–1569.

Sundström LF, Lõhmus M, Johnsson JI, and Devlin RH. 2007a. Dispersal potential is affected by growth-hormone transgenesis in coho salmon (*Oncorhynchus kisutch*). Ethology. 113: 403–410.

Sundström LF, Lõhmus M, Tymchuk WE, and Devlin RH. 2007b. Gene-environment interactions influence ecological consequences of transgenic animals. Proc Nat Acad Sci USA. 104: 3889–3894.

Sundström LF, Tymchuk WE, Lõhmus M, and Devlin RH. 2009. Sustained predation effects of hatchery-reared transgenic coho salmon *Oncorhynchus kisutch* in semi-natural environments. J Appl Ecol. 45: 762–769.

Sundt-Hansen L, Sundström LF, Einum S, Hindar K, Fleming IA, and Devlin RH. 2007. Genetically enhanced growth causes increased mortality in hypoxic environments. Biol Lett. 3: 165–168.

Sundt-Hansen L. 2008. Cost of Rapid Growth in Salmonid Fishes. PhD Dissertation. Norwegian University of Science and Technology, Trondheim.

Sundt-Hansen L, Neregård L, Einum S, Höjesjö J, Björnsson BT, Hindar K, Økland F, and Johnsson JI. 2009. Growth enhanced brown trout show increased movement activity in the wild. Funct Ecol. 23: 551–558.

Thodesen J, Grisdale-Helland B, Helland SJ, and Gjerde B. 1999. Feed intake, growth and feed utilization of offspring from wild and selected Atlantic salmon (*Salmo salar*). Aquaculture. 180: 237–246.

Thorstad EB, Fleming IA, McGinnity P, Soto D, Wennevik V, and Whoriskey F. 2008. Incidence and impacts of escaped farmed Atlantic salmon (*Salmo salar*) in nature. NINA Special Report 36. 110pp.

Twyman RM. 2005. Gene transfer to animal cells. Garland Science/BIOS Scientific Publishers, New York.

Tymchuk WEV, Abrahams MV, and Devlin RH. 2005. Competitive ability and mortality of growth-enhanced transgenic Coho salmon fry and parr when foraging for food. Trans Am Fish Soc. 134: 381–389.

Tymchuk WE, Biagi C, Withler R, and Devlin RH. 2006. Growth and behavioral consequences of introgression of a domestic aquaculture genotype into a native strain of coho salmon. Trans Am Fish Soc. 135: 442–455.

Utter F and Epifanio J. 2002. Marine aquaculture: genetic potentialities and pitfalls. Rev Fish Biol Fish. 12: 59–77.

USFDA. 2009. Guidance for industry #187— Regulation of genetically engineered animals containing heritable recombinant DNA constructs. http://www.fda.gov/cvm/GEAnimals.htm

Venugopal T, Anathy V, Kirankumar S, and Pandian TJ. 2004. Growth enhancement and food conversion efficiency of transgenic fish, *Labeo rohita*. J Exp Zool. 301: 477–490.

Waples RS. 1999. Dispelling some myths about hatcheries. Fisheries. 24: 12–21.

Waples RS. 1991. Genetic interactions between hatchery and wild salmonids: lessons from the Pacific Northwest. Can J Fish Aquat Sci. 48: 124–133.

Weber ED and Faush KD. 2003. Interactions between hatchery and wild salmonids in streams: differences

in biology and evidence for competition. Can J Fish Aquat Sci. 60: 1018–1036.

Weir LK and Grant JWA. 2005. Effects of aquaculture on wild fish populations: a synthesis of data. Environ Res. 13: 145–168.

Weir LK, Hutchings JA, Fleming IA, and Einum S. 2004. Dominance relationships and behavioural correlates of individual spawning success in farmed and wild male Atlantic salmon, *Salmo salar*. J Anim Ecol. 73: 1069–1079.

Weir LK, Hutchings JA, Fleming IA, and Einum S. 2005. Spawning behaviour and success of mature male Atlantic salmon (*Salmo salar*) parr of farmed and wild origin. Can J Fish Aquat Sci. 62: 1153–1160.

White EM, Wilson JC, and Clarke AR. 2006. Biotic indirect effects: a neglected concept in invasion biology. Diversity Distrib. 12: 443–455.

Wolf JB, Brodie ED III, and Wade MJ (eds.). 2000. Epistasis and the Evolutionary Process. Oxford University Press, Oxford.

Yamamoto T and Reinhardt UG. 2003. Dominance and predator avoidance in domesticated and wild masu salmon Oncorhynchus masou. Fish Sci. 69: 88–94.

Yaskowiak ES, Shears MA, Agarwal-Mawal A, and Fletcher GL. 2006. Characterization and multigenerational stability of the growth hormone transgene (EO-1α) responsible for enhanced growth rates in Atlantic salmon. Transgenic Res. 15: 465–480.

Part 9
Ethical Issues

Chapter 22

Aquaculture Ethics in the Biotechnology Century

Lyne Létourneau

INTRODUCTION

For several years now, I have been interested in the ethical issues raised by agricultural biotechnologies, which can be defined as a set of techniques that use living matter and biological functions to produce or transform organic molecules for agronomic activities (Guerin-Marchand 1997). After discussing animal protection law and animal ethics (Letourneau 2003), I turned my attention to the genetic modification of animals through direct manipulation of the genetic information contained in their genomes (Letourneau 2005). Next, I expanded this study to genetically modified plants (Richard and Letourneau 2006). I have now turned to fish, as biotechnological innovations in aquaculture are said to possess major transformative power. In this context, I read a number of books, articles, expert reports, and other documents that examined the ethical aspects of agricultural biotechnologies. However, despite the fact that I found some excellent discussions of these issues, my conclusions from this research are rather ambivalent. Among the reasons for this perception is my general and persistent feeling that the overviews dealt mainly with the factual components of the debate, while the principles and values underlying controversy remained unclear. If factual components remain the fundamental data upon which the development of aquacultural biotechnologies should be considered, it is not adequate to be limited to just the facts in the context of an ethical discussion. The facts must serve as a springboard to the recognition of the normative dimensions that constitute the substance of ethical dilemmas.

In the following pages, I will travel along the winding intellectual path that leads from facts to a sphere of values and principles, using the example of aquaculture biotechnology. My aim is to shed light on the parameters that constitute aquaculture ethics in the biotechnology century. Without claiming to be exhaustive, I will make every effort to create a broad picture of this area of ethical thought.

In order to achieve this goal, I will first define the notion of "ethical dilemma." To be sure, when faced with a decision to make regarding some specific action, situation, institution, or practice, the first question consists of asking oneself whether an ethical dilemma exists. Second, since the answer to this question cannot overlook the facts, I will give a summary outline of the factual aspects of the debate surrounding modern industrial aquaculture. For reasons that I will indicate, reflecting upon aquaculture biotechnologies cannot be dissociated from the issue of the intensification of our modes of food production. Consequently, modern aquaculture becomes

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

the focal point, instead of aquaculture biotechnologies in and of themselves. The factual attributes that will be described summarize the main considerations that are proffered in order to defend the development of the modern aquaculture industry, along with the arguments raised against the progress of this industry. In conclusion, the picture presented will provide the elements that are required in order to situate the ethical dilemma raised by modern industrial aquaculture.

DOES AQUACULTURE BIOTECHNOLOGY RAISE AN ETHICAL DILEMMA?

According to Metayer (2008), "ethics essentially considers action" and "affects our concern about doing what is 'right', making the 'right' decision." Ethics also deals with the distinction between good and bad. However, taking the "right" action or making the "right" decision does not consist of choosing between what is good or bad. For if there is consensus as to what is right and what is wrong, or in other words, if what is good or bad is already known, then the path that must be followed is clear; there is no ambiguity with respect to the action that must be taken or the decision that must be made. That action or decision must correspond to what is known to be right.

Of course, it is possible that, despite knowing what is right, and hence wherein lie their duty, individuals might be subject to incentives (e.g., financial rewards, conflict of interest, exercise of power, leniency on the part of employers) that cause them to breach their obligations. James calls these situations "Type II ethical problems," which he defines (2003) as

> Created by environmental or institutional incentives that give individuals opportunities to increase their utility by not conforming to ethical standards or by lowering their moral codes or ethical sensitivities. . .

To illustrate, let us take the hypothetical example of an aquaculture producer who, in violation of the law or the recommendations expressed in a code of good practices, persists in using certain products that are known to be toxic to human health, because there exists no strict mechanisms of control and such use of chemicals generates greater profit returns.

Contrary to James, I do not believe that, strictly speaking, such situations pose *ethical* problems. Although they may be regrettable and unfortunate, type II problems are *practical* problems, since they require a strategic solution to resolve them (Metayer 2008). As James (2003) states

> To be most effective, the primary focus in Type II problems should be on identifying what institutional characteristics provide incentives for people to engage in unethical activity and how those incentives could be reduced or removed. . .

Type II problems require a tactical solution that is intended to ensure compliance with the ethical standard that is established and recognized as such, whether specifically "by creating rewards to compliance with the ethical principle or by creating sanctions that will result if the ethical norm is violated" (James 2003). Solidly anchored in the objective of effectiveness, type II problems initiate practical thinking, not ethical decision-making.

It follows that the desire to take the "right" action or to make the "right" decision does not come from a supposed conflict between *doing what is right* and *doing what is wrong*. Rather, the challenge posed by ethical decision-making results from the existence of a situation that confronts the person who must act or decide with the difficult choice between *doing what is right* and *doing what is right*, where each alternative has its own disadvantages, hence the considerable uncertainty surrounding any choice to be formulated and the controversial aspect of any choice expressed (Regan 2009). In this respect, the popular saying "damned if you do, damned if you don't" illustrates perfectly the definition of a dilemma as it occurs in ethics.

Consider for instance, the following much discussed hypothetical example of a woman who is near death from a rare type of cancer, and whose husband cannot afford the drug that doctors think might save her. In order to save his wife's life, should the husband steal the drug from the druggist who discovered the drug, thereby "taking something that is not rightfully his, breaking the law, and risking possible arrest and punishment?" Or should the

husband accept the druggist's decision not to let him have the drug cheaper or pay later, in this manner preventing his wife from trying a drug that might save her life (Regan 2009)? Caught between *doing what is right* (e.g., abiding by his special duties to his wife, including his duty to help her when she is sick) and *doing what is right* (e.g., complying with the druggist's legal right to property and moral right to dispose of his property as he sees fit), the husband faces an ethical dilemma: he is confronted with the difficult choice of determining which of the two alternatives holds a greater ethical claim on him (Regan 2009).

Do aquaculture biotechnologies raise an ethical dilemma? Does their development force us, as members of society, to make a difficult choice between *doing what is right* and *doing what is right*?

MODERN AQUACULTURE AS A FOCAL POINT

Ethical dilemmas "do not rise out of thin air; without exception they are framed by a more or less complicated factual background" (Regan 2009). In the case at hand, one starting point consists in defining the range of applications designated by the term "aquaculture biotechnologies."

The various chapters of this book *Aquaculture Biotechnology* thoroughly describe these applications, and therefore, it would be futile to begin a detailed discussion here. In light of this, I will simply make a few general observations. Without a doubt, the most obvious observation is that the applications relate to aquaculture, which is defined as "the entire set of aquatic animal breeding and aquatic animal or plant farming activities" (Knockaert 2006). In these chapters, the emphasis is placed primarily on fish and shrimp. Among the biotechnologies examined, some are intended to acquire new knowledge (e.g., advanced knowledge of fish host–pathogen, information about relevant biological processes), while others offer solutions to practical problems (e.g., DNA vaccines for viral diseases). Finally, there are also biotechnologies that deal with the genetic manipulation of fish (e.g., germ-cell transplantation, regulation of transgene expression), which include examples of opportunities in that field (e.g., freeze-resistant salmon). With a few exceptions (i.e., environmental monitoring, xenotransplantation, and cryopreservation), all of the biotechnologies that are described are part of an effort to pursue the objectives perceived by researchers as likely to promote the productivity and profitability of the aquaculture industry, such as broodstock improvement, disease and reproduction control, growth enhancement, feed conversion efficiency, and temperature and salinity tolerance. As Aerni (2004) points out

> Aquatic biotechnology is the latest step in the technological evolution of modern aquaculture. As in many other research areas of the life sciences, modern biotechnology is considered to be a new tool to improve the quality and quantity of fish reared in aquaculture.

This voluntary alignment of scientific research with the interests of the aquaculture industry is not insignificant, because it mirrors the development model that has been used for intensive milk, meat, and egg production. As Knockaert (2006) points out, "the [aquaculture] approach is comparable to that of agriculture, which attempts to control environments and the food cycle (fertilization) and the species (domestication)." In fact, "aquaculture is now undergoing and will continue to undergo a revolution . . . similar to that which has driven improvement in agricultural stock animals for the past few hundred years" (Maclean 2003). According to the generally accepted definition, "intensive livestock husbandry" refers to the use of methods that result "in the rapid production of animal products by standardized methods involving economy of land and labour" (Brambell 1965). In the industrial version of intensive livestock husbandry, the methods used are capable of exploitation on a large scale and involve a high degree of mechanization and automation (Brambell 1965). Having become a highly capitalized industry, today's aquaculture is characterized by its technically specialized conditions and uptake of innovative production and management technologies, resulting in increased productivity (Aerni 2004). Therefore, it can be concluded that "[m]odern aquaculture is truly an industrialized food process" (Millar and Tomkins 2007). As a result, just like this is the case for intensive agriculture (animal and plant), the merits of aquaculture are being called into question more and more frequently. Criticisms include and extend

to biotechnology innovations that are developed to serve industry.

Consequently, although some argue that consumers are likely to increasingly accept "fish transgenic for sequences from fish origin rather than those, for example, of mammalian origin" (Maclean and Laight 2000), such comments disregard the fact that, beyond the usually heated debate over genetic manipulation of animals and plants (which the use of constructs that contain only fish-derived sequences attempts to answer), there is the much wider and more menacing backdrop of the dispute over the intensification of our food production methods. To be sure, even if one day consumers' attitudes were to show widespread support toward genetic engineering and biotechnology generally, which is currently not the case (Logar and Pollock 2005), the war that is being waged against intensive agriculture, and modern aquaculture in the same vein, would still not lack ammunition and could continue. This being the case, my discussion of the relevant facts in the context of aquaculture biotechnologies will involve aquaculture production in general, as it is currently practiced around the world at the industrial scale.

FEATURES OF A CONTROVERSY

Since the dawn of humanity, marine animals have made a substantial contribution to the protein that forms an integral part of human nutrition (Hastein 2004; Millar and Tomkins 2007). Traditionally, these animal proteins were made available through the fishing industry. However, with the rapid rise in world population, the overexploitation of marine resources, and the deterioration of certain aquatic environments, this industry "has experienced a levelling off and a decline in exploitable biomasses in recent decades" (Knockaert 2006). However, although global fish consumption is expected to reach the level of approximately 150 million tons per year around 2030 (Knockaert 2006), the maximum support capacity of the oceans is estimated at 90 million tons per year (Vinatea 2004). As a result, the annual demand for seafood products will exceed the capacity of the fishing industry by approximately 60 million tons.

In this context, aquaculture is perceived as *the* solution that will make it possible to palliate the growing demand for seafood products and preserve food security for humanity in the twenty-first century. With a nod to the "green revolution" of the 1960s, which resulted "in aggregate—though very unevenly across space—an extraordinary track record of sustained global agricultural productivity/output increase" (Buttel 1995), some authors have termed this faith in modern aquaculture the "blue revolution" (Costa-Pierce 2002). There is no question that aquaculture has evolved significantly over the last three decades, and with an average growth rate of more than 8% per year, it has become the fastest growing animal food production industry in the world (Vinatea 2004).

A number of benefits have been attributed to the modern aquaculture industry, including the following:

- Helping to provide a continuous global supply of seafood,
- Compensating for the leveling off of fisheries and decreasing the pressure exerted on halieutic resources by commercial fishing, and
- Helping to drive prices down, which is primarily of benefit to consumers, and which facilitates and even guarantees access to food that is recognized for its nutritional value and its contribution to a balanced diet.

The aquaculture industry is also claimed to help in ensuring economic development in rural areas by providing jobs and a stable source of income for members of communities in need and communities that are faced with major structural changes linked to changes in agriculture. In light of this, modern aquaculture is promoted "as a notable force for social development" (Millar and Tomkins 2007).

Between November 2005 and June 2006, interviews were conducted with some 20 stakeholders in the Quebec aquaculture sector (i.e., fish breeders and farmers, government officers and regulators, support organizations, university researchers, and nongovernmental organizations such as citizen and environmental groups) in an effort to ascertain their perceptions of the realities, challenges, and issues facing the industry. The respondents echoed the aforementioned discourse on the benefits of aquaculture around the world (Jean and Letourneau 2007).

Still, despite the fact that modern aquaculture promises significant benefits, the rapid growth and

P1: SFK/UKS P2: SFK
BLBS089-c22 BLBS089-Fletcher October 3, 2011 12:0 Trim: 246mm X 189mm Printer Name: Yet to Come

production methods used raise a number of concerns, including in the court of public opinion, where there is an increasing awareness of the risks and potential negative impacts that may be caused by industry activities. During the abovementioned interviews, many potentially harmful effects were identified in connection with aquaculture in Québec, and on a national and international scale, reflecting the common criticisms pertaining to aquaculture. From the outset, a number of producers voiced the opinion that it was somehow "easier to highlight the negative aspects of the industry [than the positive ones]!"

In general, three categories of potentially harmful effects were cited in the following order: environmental impact; the difficulties associated with cohabitation between aquaculture and tourism, housing, logging, or fishing in coastal areas; and the effects on human health and the welfare of fish (Jean and Letourneau 2007).

It is hardly surprising that the stakeholders who were questioned have placed the impact on the environment at the top of the list of the harmful effects of aquaculture. As revealed in the following statements by Millar and Tomkins (2007), this is a central theme in the debate surrounding modern aquaculture, especially in light of the possibility that genetically modified fish may one day be the subject of commercial production:

> A number of aquaculture problems have been identified. Significant concerns have been expressed about: (i) the potential risks associated with environmental impacts from the increased use of pesticides and antibiotics; (ii) discharge of production wastes and pollutants; (iii) ecological impacts from any potential escapees; (iv) frequency and diversity of disease outbreaks; (v) increased wetland and land use impacts; and (vi) reliance on high-energy inputs (such as aquafeeds). With the rapid expansion of production, there are increasing concerns that production efficiency will be the dominant driver for the industry, at the cost of environmental protection and social justice.

Of course, the magnitude of the environmental impact of aquaculture will vary between different aquaculture systems and different types of fish (Aerni 2004). In addition to these factors, there is a geographical aspect linked to the location of the facilities, and therefore, to the hydrography of the site of the farming facility (Belias 2003).

In a relatively recent article, Nash (2003) offers the synthesis of five literary reviews that discuss the environmental impact of Atlantic salmon farming in the Puget Sound region, which is a seaway in the Northwestern United States that leads to the Pacific Ocean. According to him, two issues appear to carry the most risk. These are the impact on the sediments beneath net-pen farms from biodeposits (i.e., feces and unconsumed food) and the accumulation of heavy metals like zinc and copper (Nash 2003). Among others, he identifies the increased incidences of disease among wild fish as carrying a low risk and points toward the escape of a nonnative species (with its concomitant possible hybridization of such specimens with other salmonids, colonization of salmonid natural habitat, competition with native species for forage, predation on indigenous species, and introduction of exotic pathogens) as involving very little or no risk (Nash 2003).

However, it is too soon to reach any definitive conclusions concerning the nature and scope of the environmental impact of industrial aquaculture at this stage. The question remains open and continues to be a stimulus for major scientific research, including in such areas as the positive, negative, or neutral effects of the potential introduction of transgenic fish into the "free" environment.

In any case, my objective here is not to tout the positive or negative claims made with respect to modern aquaculture, whether they involve the environment, social development, or other issues. In other words, my goal is not to decide which claims are true and which are false. Such an undertaking would require me to complete a considerable amount of work that goes beyond the framework of this study.

Instead, my interest lies in noting these various assertions, because through them, much of the factual context that frames and feeds the controversy surrounding industrial agriculture can be described. Table 22.1 provides a summary of these main factual elements, depending on whether they support aquaculture or oppose it.

Table 22.1. Factual Context.

For	Against
Maintaining seafood supply	Harmful effects on environment
Less pressure on fishing	Conflicts involving use
Access to lower prices for healthy food	Negative effects on human health
Contribution to the economic development of rural regions	Negative impacts on the welfare of farm fish

That being said, it is important to note that proper decision-making in ethics involves giving the best reasons, or arguments, in support of one course of action versus another (Fraser 2007). Therefore, it is necessary to verify the accuracy of factual claims as an integral part of evaluating any ethical argument (Russow 2002a). For if empirical premises appear to be erroneous, or if they are not yet fully proven, then this will have an impact on the soundness or reliability of this ethical argument.

In the context of aquaculture, this means that no relevant fact included in Table 22.1 can immediately be taken for granted or assumed to be true. The accuracy of each factual claim mentioned in Table 22.1 must be provable in order for the ethical arguments, which invoke them to be recognized as sound or reliable, and as a result, to bear up under the weight of critical analysis and decision-making process.

FROM FACTS TO ETHICAL ISSUES

Describing the facts that provide a structure for a given controversy is not the same as depicting the ethical dimensions specific to this controversy. As stated in the introduction, ethics deals with the sphere of principles and values. More specifically, it refers to higher standards (i.e., principles) or ideals (i.e., values) that we, or the person who is putting forth the argument, recognize as valid and feel an obligation to uphold through our actions or decisions (Metayer 2008). On the basis of the principles or values, ethical arguments "typically support conclusions that claim that someone *ought* or *ought not* do something, or that a certain sort of action is either *right* or *wrong*, or that a certain sort of thing has *positive* (goodness), or *negative* (badness) moral value" (Russow 2002a). Consequently, ethical arguments are inextricably linked to a *normative* component. That is precisely the feature that distinguishes ethical arguments from other arguments (e.g., political, legal, economic). As Russow (2002a) explains

> Two features are distinctive of moral reasoning. The first is that a complete moral argument will almost always involve at least one premise that makes a factual or empirical claim, and at least one that appeals to a general moral principle [or value statement].

In addition to empirical premises, ethical arguments include normative premises that refer to principles or values. It is through these normative premises that the domain of ethics, strictly speaking, takes shape, because without normative premises, or in other words, higher requirements imposed by principles and values, there are no ethics.

The summary (Table 22.1) presented in the previous section clearly shows that, as deployed in the marketplace of ideas, the debate surrounding modern aquaculture proposes an opposition between social and environmental development, between consumption and animal welfare, etc. According to the terms of this polemic, we are confronted by a dilemma where we must choose between supporting the growth of industrial aquaculture on the one hand, and thus giving priority to social development over the environment, to consumer interests over the interests of farm fish, etc., or on the other hand, ending the aquaculture industry as we know it in its modern version, and thus giving priority to protection of the environment over a continuous global supply of seafood products, to animal welfare over growth in rural regions, etc.

This multifaceted tension surrounding modern aquaculture is clearly evidence of a dilemma. But

what are the ethical dimensions of this dilemma? Between which "good" are we forced to choose when contemplating the acceptability of the aquaculture industry?

The answer to that question depends on the normative premises that are invoked upon the debate—either explicitly or implicitly—by those who participate in it, whether directly, as stakeholders sharing their opinions in the public sphere or political arena, or indirectly, as members of expert committees or through the publishing of articles in academic journals.

Let us consider the issue of the environmental impact of industrial aquaculture. Several schools of thought propose their respective concepts of the relationship between human beings and nature. Some philosophers advocate a noninstrumental view of nature, according to which the environment (e.g., mountains, rivers, species of animals or plants, ecosystems, wilderness, nature in its entirety) is valuable in itself and deserves to be protected independently of any potential benefit to human beings, while other thinkers defend an instrumental view of nature, according to which the environment cannot be the subject of direct moral concern, such that its protection is dependent on the defense of human interests (Des Jardins 1995; Russow 2002b). These diametrically opposed concepts of the moral status of the environment correspond with distinct normative premises that result in a different assessment of the facts when applied concretely.

First, let us consider the duty to preserve the stability, integrity, and beauty of the land (Leopold 1949), which reflects a noninstrumental concept of nature. Any ethical argument based on this normative premise will attempt to criticize the potentially harmful effects of modern industrial aquaculture on the environment as violating the duty just mentioned. For it is through the lens framed by the three criteria of stability, integrity, and beauty that the environmental effects of aquaculture will be analyzed, and judged unfavorably.

Second, let us examine the principle of the "happiness of the greatest number" (Bentham 1879), which refers to an instrumental perspective of nature. A strict interpretation of this utilitarian principle, which was popular among economists, consists in reducing all human interests to their monetary significance (Metayer 2008). When applied to aquaculture, such an interpretation of the principle would authorize the installation of fish farms in a given region, regardless of the harmful effects on the environment, provided that such a project would generate more economic benefits than those generated by preserving the region in its natural state. On the basis of such a principle, assessing environmental consequences is thus of no practical interest. Indeed, because such an interpretation of the utilitarian principle does not assign nature any value distinct from profit, the action of humans in nature is evaluated exclusively by applying the economic criterion.

Fortunately, damage to the environment that may have been authorized in the past by such cost–benefit analyses is likely to be avoided these days by applying the principle of sustainable development. Although firmly entrenched in an instrumental view of nature, this principle offers a reformulation of the utilitarian principle that allows for the exploitation of nature in order to meet current needs, provided that future generations are not prevented from meeting their needs as a result (World Commission on Environment and Sustainable Development 1987). This condition sets the evaluation criterion of environmental impact.

Following the example of the duty to preserve the stability, integrity, and beauty of the earth, the utilitarian principle and the principle of sustainable development express distinct normative criterion according to which individuals who subscribe to them will evaluate the body of facts related to the impact of modern aquaculture on the environment.

In bringing together diverse understandings of the relationship between human beings and nature, environmental ethics is expressed in a pluralistic way. Similarly, several schools of thought propose their respective visions of how to manage the conflicts that occur among users of natural resources (Thompson 2002; Schmidtz and Willott 2003). Various perspectives are applied to the world's food situation (LaFollette 2003), future generations (Wolf 2003), the required treatment of animals and fish (DeGrazia 1996), and the social role of agriculture (Taliaferro 2002), etc.

All in all, there are a variety of normative premises related to each aspect of the factual context specific

to modern aquaculture—or at least insofar as its main elements are concerned. That being the case, a great many ethical arguments can be put forward. While some arguments will stress the best balance of consequences (consequentialist ethics), others will be based on duty (deontological ethics) or propose a criterion of excellence of character or society (virtue ethics).

It would not be possible to review all of relevant normative premises here. Still, for our immediate purposes, the three following points can be made:

- First, there is a plurality of normative premises in the debate surrounding modern industrial aquaculture.
- Second, all of these normative premises crystallize around certain *key ethical issues* (e.g., the moral status of the environment, our moral responsibility to help those in need, the notion of intergenerational justice, the moral treatment of animals) that transcend the context of the aquaculture industry. To be sure, other controversial situations do provoke these same issues. Examples include intensive animal husbandry, genetically modified crops, commercial seal hunting, and biofuels. Extending across several areas, these *key ethical issues* are therefore transverse in nature.
- Finally, it is important to remember that it is the specific positions that one adopts with respect to these *key ethical issues* that determine one's outlook on aquaculture.

Considering the aforementioned, it follows that the ethical dimensions of the dilemma raised by modern aquaculture refer to a number of *key ethical issues* that seek to clarify the general moral requirements of life in society and within nature. As such, in the biotechnology century, there is thus no aquaculture ethics that exists outside of those fundamental issues. There are only facts particular to this industry.

CONCLUSION

In the preceding pages, my objective has been to follow the intellectual path that leads from facts to the sphere of values and principles, in order to define the constituent parameters of modern aquaculture ethics. A number of milestones have been erected along the way in terms of providing theoretical or conceptual clarification, whether involving the concept of "ethical dilemma," the role played by facts in ethical thinking, the distinction between empirical claims and normative statements, proper decision-making in ethics as involving the assessment of factual claims, or the inextricable link between appreciation of facts and principles or values.

All of these elements combine to generate a concept of ethics, or more specifically, a concept of ethical decision-making that seeks to reach rational objectivity without claiming any possibility for absolute truth. In a way, taking into consideration the fact that members of the scientific community will probably account for the majority of readers of this work, I guess that my undeclared goal was also to illustrate that ethics is not intended to be a hold-all, but that it contains analysis tools that can shed light on the delicate transition that must be operated from the preliminary step corresponding to the description of facts to the next level represented by the identification of principles and values, which really relate, as I have said multiple times, to what ethical thinking is all about.

ACKNOWLEDGMENTS

This paper was written as an integral part of a research project that is directed by Grant Vandenberg from the Animal Science Department at Laval University (Quebec, Canada), funded by the Natural Sciences and Engineering Research Council of Canada (NSERC). The author wishes to express her sincere thanks to both for their financial support.

REFERENCES

Aerni P. 2004. Risk, regulation and innovation: the case of aquaculture and transgenic fish. Aquat Sci. 66: 327–341.

Belias CV. 2003. Environmental impacts of coastal aquaculture in eastern mediterranean bays: the case of Astakos Gulf, Greece. ESPR. 5: 287–295.

Bentham J. 1879. An Introduction to the Principles of Morals and Legislation. Clarendon Press, Oxford.

Brambell FWR, Chairman. 1965. Report of the Technical Committee to Enquire into the Welfare of Animals Kept under Intensive Livestock Husbandry Systems. HMSO, London. Cmnd 2836.

Buttel FH. 1995. The global impacts of agricultural biotechnology: a post-green revolution perspective. In: Issues in Agricultural Ethics (eds. Mepham TB,

Tucker GA, and Wiseman J). Nottingham University Press, Nottingham. pp 345–360.

Costa-Pierce BA. 2002. The "blue revolution"—aquaculture must go green. World Aquacult. 33: 4–5.

DeGrazia D. 1996. Taking Animals Seriously—Mental Life and Moral Status. Cambridge University Press, Cambridge.

Des Jardins JR. 1995. Éthique de l'environnement—Une introduction à la philosophie environnementale. trad. Nguyen V-D and Samson L. Presses de l'Université du Québec. Sainte-Foy.

Fraser J. 2007. Ethics. In: The Internet Encyclopedia of Philosophy (eds. Fieser J and Dowden B). WordPress, the University of Tennessee, Martin. http://www.iep.utm.edu/e/ethics.htm.

Guerin-Marchand C. 1997. Les manipulations génétiques. Presses universitaires de France. Paris. coll. Que sais-je, no. 3152.

Hastein T. 2004. Animal welfare issues relating to aquaculture. Proceedings of the Global Conference on Animal Welfare: An OIE Initiative. World Organization for Animal Health, Paris. pp 219–227.

James HS Jr. 2003. On finding solutions to ethical problems in agriculture. J Agr Environ Ethic. 16: 439–457.

Jean D and Letourneau L. 2007. Aquaculture, éthique et société : évaluation de la prise en compte de la notion de responsabilité sociale par l'industrie aquacole québécoise—Résumé synthèse. http://raq.uqar.qc.ca/rapports_publics/rapport_final_jean_letourneau.pdf.

Knockaert C. 2006. Salmonidés d'aquaculture—De la production à la consommation. Éditions Quae. Versailles.

LaFollette H. 2003. World hunger. In: A Companion to Applied Ethics (eds. Frey RG and Wellman CH). Blackwell Publishing, Oxford. pp 238–253.

Leopold A. 1968. 1949. A Sand County Almanac, and Sketches Here and There. Oxford University Press, London.

Logar N and Pollock LK. 2005. Transgenic fish: is a new policy framework necessary for a new technology? Environ Sci Policy. 8: 17–27.

Letourneau L. 2003. Toward animal liberation? A look at the new anti-cruelty provisions in Canada and their impact on the moral status of animals. Alberta Law Rev. 40: 1041–1055.

Letourneau L. 2005. The regulation of animal biotechnology: at the crossroads of law and ethics. In: Crossing Over—Genomics in the Public Arena (eds. Einsiedel EF and Timmermans F). University of Calgary Press, Calgary. pp 173–192.

Maclean N. 2003. Genetically modified fish and their effects on food quality and human health and nutrition. Trends Food Sci Technol. 14: 242–252.

Maclean N and Laight RJ. 2000. Transgenic fish: an evaluation of benefits and risks. Fish Fish. 1: 146–172.

Metayer M. 2008. La philosophie éthique: enjeux et débats actuels. 3e éd. ERPI, Saint-Laurent.

Millar K and Tomkins S. 2007. Ethical analysis of the use of GM fish: emerging issues for aquaculture development. J Agr Environ Ethics. 20: 437–453.

Nash CE. 2003. Interactions of Atlantic salmon in the Pacific Northwest: VI. A synopsis of the risk and uncertainty. Fish Res. 62: 339–347.

Regan T. 2009. Research Ethics: An Introduction. In Research Ethics Mini Rounds – Module 1 (eds. Kriesberg N and Regan T). North Carolina State University Research & Professional Ethics Program. http://www.ncsu.edu/grad/research-ethics/modules/regan.htm.

Richard V and Letourneau L. 2006. Portrait du questionnement éthique entourant la transgenèse des animaux et des plantes. In Bio-ingénierie et responsabilité sociale (ed. Letourneau L). Éditions Thémis. Montréal. coll. ⟨Droit, biotechnologie et société⟩. pp 13–55.

Russow L-M. 2002a. Reasoning. In: Life Science Ethics (ed. Comstock GL). Iowa State Press, Iowa. pp 33–66.

Russow L-M. 2002b. Environment. In: Life Science Ethics (ed. Comstock GL). Iowa State Press, Iowa. pp 93–123.

Schmidtz D and Willott E. 2003. The tragedy of the commons. In: A Companion to Applied Ethics (eds. Frey RG and Wellman CH). Blackwell Publishing, Oxford. pp 662–673.

Taliaferro C. 2002. Farms. In: Life Science Ethics (ed. Comstock GL). Iowa State Press, Iowa. pp 225–252.

Thompson P. 2002. Land. In: Life Science Ethics (ed. Comstock GL). Iowa State Press, Iowa. pp 169–189.

Vinatea T. 2004. Aquaculture philosophy. World Aquacult. 35: 62–63.

Wolf C. 2003. Intergenerational justice. In A Companion to Applied Ethics (eds. Frey RG and Wellman CH). Blackwell Publishing, Oxford. pp 279–294.

World Commission on Environment and Sustainable Development. 1987. Our Common Future. Oxford University Press, Oxford.

Index

Note: Italicized page locators indicate a photo/figure; tables are noted with *t*.

A
Abalone, dispersed *Sall* repeat in, 73
Abiotic stress responses, shrimp aquaculture and, 25
Acanthopterygii Perciformes, examples of species with genomic resources and number of ESTs in GenBank, 4*t*
Acipenserids, cell lines from ATCC available for, 207
actin, RT-PCR analysis of, *202*
Actions, ethics and, 346
Activators, in inducible gene expression systems, 244, 246
Activin, 293
Activin receptor signaling pathway, TGF-β ligands and, *292*
Activin type I and II transmembrane receptors, 292
ActRIIA receptors, 292
ActRIIB receptors, 292, 293
Acute phase serum amyloid A, 213*t*
Adaptive immune response, antigen presentation in, 125–126
Adjuvants, challenge for effective vaccines and, 128
Adrianichthylidae, medaka within, 268
Aeromonas hydrophila
genomic sequencing of, 90*t*
transgenic expression and resistance against, 112
Aeromonas salmonicida
bacterial pathogens and genomic variability and subspecies identification, 91–92
proteomics, 92–93
host metabolomics and, 96–97
host-pathogen interactions and, 94
SSH cDNA libraries and study of, 95
vaccines for, 153
Aeromonas salmonicida subsp. *salmonicida,* genomic sequencing of, 90*t*
AE6, DNA vaccine vector construction and, 160
Affymetrix, 6, 13
AF(G)P, 254
binding of, to ice, 255
AFGP, Types I-IV, isolating from fish, 254*t*
AFLPs. *See* Amplified fragment length polymorphisms
AFP concentration, comparison of type 1 skin, liver, and hyperactive AFP antifreeze protein activities as function of, *255*
AFPs. *See* Antifreeze proteins
African catfish *(Clarias gariepinus),* ActRIIB protein injection and increased size of, 294
Agilent, 6
Agricultural biotechnologies, ethical issues around, 345
AHNV. *See* Atlantic halibut nodavirus
Albumin, DNA vaccine vector construction and, 159
Alginates, 110
All-female broodstocks
of Atlantic halibut, production of, 44–46
gonadal differentiation and endocrine sex reversal, 45–46
sex determination and gynogenesis, 45
Alpha-helical antimicrobial peptides, 106
Alternative protein sources, physiological factors related to use of, 62
American oysters *(Crassostrea virginica),* centromeric sequences identified in, 73
American Tissue Culture Collection, 207
Ammonia, marine aquaculture and accumulation of, 76
Among-family selection, aquaculture and, 50

Aquaculture Biotechnology, First Edition. Edited by Garth L. Fletcher and Matthew L. Rise.

Amplified fragment length polymorphisms
genetic mapping and, 60
pedigree analysis and, 46
penaeid species and, 35
AMPs. *See* Antimicrobial peptides
Amyloodinium ocellatum, piscidin 2 active against, 110
Androgenesis
clonal lines and, 56
homozygous clones and, 59
Androgens, indirect feminization and, 45
Animal ethics, 345
ANNs. *See* Artificial neural networks
Antarctic fish, antifreeze glycoproteins in serum of, 253
Antibiotics, xi
aquaculture and increased use of, 349
Antibody arrays, 15
Antifreeze protein freeze resistance strategies, winter flounder as model for, 255–258
Antifreeze protein gene transfer, 253–263
research progress to date, 259–260
Antifreeze proteins, 253–255
binding to ice, *255*
cold marine regions and fish protected with, 253
embryo cryopreservation and, 311
increasing functional levels of, 260–263
isolated from fish, 254*t*
protein engineering and enhancing activity of, 262, *262*
seasonal changes in, in Newfoundland winter flounder, 256, *257*
Type I, 254*t,* 256, 260, 261, 262, 263
Type II, 254*t*
Type III, 254*t,* 260, 261, 262, 263
Type IV, 254*t*
Antigen presentation, in adaptive immune response, 125–126
Antigen-presenting cells, 122
Anti-lipopolysaccharide factor, RNAi-based experiments in Penaeid shrimp and, 31*t*
Antimicrobial Peptide Database, 106
Antimicrobial peptides
activities of, 107–108, 110
antibacterial activity, 107–108
antifungal activity, 108, 110
antiparasitic activity, 110
antiviral activity, 108
cytotoxic activity, 110
immunomodulatory activity, 110
control of disease through use of, 111–113
direct administration, 111–112
DNA vaccines, 112
genetic selection for enhancement of disease responses, 112
transgenic expression, 112–113
vaccine adjuvants, 112
current approaches to control of disease in aquaculture, 110–111
distribution and spectrum of activity of, from marine fish and invertebrates, 109*t*
distribution of, 106–107
future developments of, 113
physical properties of, 105–106
production of, 105
as therapeutants in aquaculture, 105–113
therapeutic potential of, 110–113
Antiviral protection, in shrimp, RNAi as tool for promoting, 34–35
APCs. *See* Antigen-presenting cells
Apex-IHN vaccine, 98, 154
Aqua Bounty Technologies, xii
Aquaculture
clones and alternative feeds in, 61–62
defined, 347
domestication process and, 63–64
domestic selection, divergence and, 325
fish stem cell technology applications in, 196
freeze-resistant fish produced for, summary, 263
functional characterization of fish skeletal muscle cells and their application in, 216–219
future of biotechnology and immunology in, 131–132
future of shrimp genetics and genomics relative to, 37
gene constructs and, 235
"happiness of the greatest number" principle and, 351
history behind, xi–xii
pathogens identified in wastewater generated by, 76–77
potentially harmful effects of, 349
public opinion and criticisms pertaining to, 349
rapid expansion of, xi, 321–322
sea cage, low temperature limitations to, 258–259
transgenic technology and, 267–268
trout macrophage studies and impact on, 212, 214
viral diseases and, 141
Aquaculture biotechnology(ies)
ethical dilemma relative to, 346–347
genetic background, 322–323
potential ecological and genetic impacts of, 321–336
practical biological containment methods and, 336
Aquaculture broodstocks, intentional selection in, 324
Aquaculture escapees
event with, *326*
transgenesis and risks associated with, 335
Aquaculture ethics in biotechnology century, 345–352
Aquaculture fish species, selected, expressed sequence tag collections of, 4*t*
Aquaculture industry, benefits attributed to, 348
Aquaculture-relevant traits, examples of experimental approaches to, *14*
Aquaculture research, "genomics toolboxes" and, 3
Aquafeeds, 349
AquaNet, 283, 287
Aquaporins, ectopic expression of, embryo cryopreservation and, 312
Aquatic biotechnologies, defining range of applications designated by, 347
Aquatron (Dalhousie University), 285, 286
Aqueorea victoria, cloning of first *GFP* cDNA from, 275
Arabidopsis, recombinant inbred lines and, 56
Arasin, 106
Arctic charr *(Salvelinus alpinus)*
contribution of antifreeze proteins to, *256*
cosmid libraries for, 75
freezing point of (without AF(G)P), 255
thermal tolerance of, 63
Argonaute family, 187
Aromatase inhibitors, masculinizing fish with, 45

Array data, quality control of, 10
Artificial neural networks, 36, 37
Artificial response elements, 275
Astacidin 2, 106
ATCC. *See* American Tissue Culture Collection
Atlantic Canada
  Atlantic salmon farming in, 258
  development of genetic/genomic tools and production improvements in Atlantic halibut in, 44, *44*
  maximum spring sea ice conditions in, *259*
Atlantic cod *(Gadus morhua),* 3
  contribution of antifreeze proteins to, *256*
  immune-related gene discovery in, 96
  mapping genome of, 132
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
  sperm cryopreservation studies for, 307
Atlantic Cod Genomics and Broodstock Development Project, 3–4, 132
  functional genomics workflow in, *5*
Atlantic cod 20K oligonucleotide microarray, description of, 9
Atlantic halibut broodstock management
  broodstock selection, 50–51
    impact of generation time, 50
    marker-assisted selection, 50–51
  future directions, 51
  genetic and genomic approaches to, 43–51
  genetic linkage map and, 47
  overview, 43–44
  pedigree analysis and, 46–47, 48*t*
  production of all-female stocks of Atlantic halibut, 44–46
    gonadal differential and endocrine sex reversal, 45–46
    sex determination and gynogenesis, 45
  quantitative trait loci, 49–50
    considerations for QTL analysis, 49–50
    status of QTL mapping in halibut, 50
Atlantic halibut *(Hippoglossus hippoglossus)*
  analysis of expressed sequence tags in, 179
  iteroparous batch spawning by, 43–44
  maternal mRNA from mitochondrial genes of, 181
  microarray platforms for, 7*t*
  sperm cryopreservation studies for, 307
  timeline for development of genetic/genomic tools and production improvements in, *44*
Atlantic halibut nodavirus, viral DNA vaccines against, 156*t*
Atlantic salmon farming
  beginning of, 258
  environmental impact of, 349
Atlantic salmon genome sequencing project, 61
Atlantic salmon *(Salmo salar)*
  AFP genes transferred to, 253
  BAC libraries for, 75
  centromeric sequences identified in, 73
  competitive displacement of wild individuals and, 327
  contribution of antifreeze proteins to, *256*
  cytokines as adjuvants for DNA vaccines and, 161
  freezing point of (without AF(G)P), 255
  gene expression-based toxicogenomics approach applied to, 277
  genome sequencing projects for, xii
  "genomics toolboxes" and, 3
  GH and myosatellite cells from, 219
  immune system in, 121
  IPNV capsid protein injected in, 153
  length of time plasmid DNA detected in, 159
  local population structure and, 323
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
  oligo-based microarrays for, 94
  persistence and distribution of plasmid DNA in, 166
  SD locus and, 74–75
  selective crossbreeding programs for, introduction of, xi
  sibling pair produced with one hemizygous growth hormone transgenic parent, *334*
  transgene expression and transmission in, 236
  viral DNA vaccines for, 155*t*
Atlantic salmon sea cage operation (Fortune Bay, Newfoundland), *322*
Atlantic wolffish *(Anarhichas lupus)*
  AF(G)P in, 255
  contribution of antifreeze proteins to, *256*
Attenuated vaccines, 98
Autogenous vaccines, DNA vaccines *vs.,* 98
Automated DNA sequencing, xii

B
BAC end sequences, 3
Backcross lines, sexually dimorphic traits in clonal fishes and, 65
BAC libraries. *See* Bacterial artificial chromosome (BAC) libraries
BACs. *See* Bacterial artificial chromosomes
Bacterial artificial chromosome (BAC) libraries, 3
  chromosome identification in rainbow trout and, 73
Bacterial artificial chromosomes, 36, 241
Bacterial pathogens
  A. salmonicida, 89, 91–93
    genomic variability and subspecies identification, 91–92
    proteomics, 92–93
  of fish, genomic sequencing of, 90*t*
"Basal promoter," transgene expression and, 239
Bathymodiolus azoricus, 106
Bay scallops, type of karyotype in, 73
BBs. *See* Brockmann bodies
Bcl-X1, *5*
Belgian Blue cattle, myostatin gene in, 291
Beta-actin promoter, in vivo testing of, 160
Beta-catenin-dependent (or canonical) pathway, 183
Beta-catenin-independent (or non-canonical) pathway, 183
Beta-catenin-2, 183
Beta-glucans immunostimulants, 111
Betanodaviruses
  RNA interference and viral pathogenesis of, in fish, 89
  vaccine development and, 158
β-cells, juvenile-onset diabetes and loss of, 281
Beta-sheet class of AMPs, 106

β-integrin, RNAi-based experiments in Penaeid shrimp and, 31*t*
Big defensin, distribution and spectrum of activity of, 109*t*
Bioactive domain, for myostatin peptide, *293*
Biocarta, 12
Bioconductor, 10, 11
Bioconductor project, 9
Biodeposits, 349
Biodiversity
  cryopreservation and, 314
  species invasions and effects on, *326*
Biofreezers, 306
Bioinformatics, 3, 277
Biological response modifiers, 129
  antibody response after vaccination and, 130
  enhanced immune responses and, 129
Biomarkers, microarrays, toxicogenomics and, 277
Biomonitoring fish
  models of, 268–269, 271–273
    estrogenic compounds, 269, 271
    heavy metals, 271–272
    mutagens, 272–273
    persistent organic pollutants, 272
  transgenic
    developing, 267
    systems of, examples, 270*t*
Biomonitoring transgenic systems, advantages of, 274–275
Biotechnological strains, cryopreservation and preservation of, 314
Biotechnology. *See also* Ethics
  future of, in aquaculture, 131–132
  modern aquaculture production and, 322
  wide use of transgenic technology and, 267
Biotechnology community, ecologically neutral technology development and, 336
Bivalves, AMP sequences identified in, 107
Black scallop *(Mimachlamys varia),* type of karyotype in, and location for major and minor rDNA loci, 73
Black tiger shrimp *(Penaeus monodon),* AMP sequences identified in, 107
Blast database (NCBI), 13
Blastomere cryopreservation, 312–313
Blast2GO, 10*t,* 13
Blue catfish *(Ictalurus furcatus)*
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
Bluefin tuna *(Thunnus thymus),* germ cell transplantation and, 223
Blue fluorescent protein, 275
Blue revolution, 348
  eco-evolutionary considerations for, 321–336
Bonamia ostreae, tachyplesin I active against, 110
Bovine papillomavirus-1 E2 DNA-binding protein, structural similarity between VP9 and, 150
Brg1, in zebrafish, 185
BRMs. *See* Biological response modifiers
Brockmann bodies, 282
Broodstock improvement, 347
Broodstock management, cryopreservation and, 313
Broodstock selection markers, xii
Brook trout *(Salvelinus fontinalis),* primary cell culture methods for head kidney and, 208
Brown-spotted grouper *(Epinephelus tauvina),* studying proteomic profiles of, with iTRAQ and LC-MS/MS, 145, 147
B7 molecules, 125

C
Caenorhabditis elegans, feeding or bacteria-mediated RNAi and, 34
Callinectin, 106
  distribution and spectrum of activity of, 109*t*
Canadian Food Inspection Agency, 154
Canadian Institutes for Health Research, 283
Candida albicans, AMPs and, 108
Canulation, trout sperm extraction by, *309*
Captively reared populations, lower genetic diversity in, 327–328
Carcinin, distribution and spectrum of activity of, 109*t*
Carp. *See also* Common carp
  beta-actin promoter in, evaluation and in vivo testing of, 159, 160
  clonal lines of, 55
  gene expression-based toxicogenomics approach applied to, 277
  sperm cryopreservation studies for, 307
Carp myosin-heavy-chain promoter, DNA vaccine vector construction and, 160
Caspase, RNAi-based experiments in Penaeid shrimp and, 31*t*
Caspase-3 homolog, RNAi-based experiments in Penaeid shrimp and, 31*t*
Castaigne, Jean-Paul, 283
Catfish
  BAC libraries for, 75
  genome sequencing projects for, xii
  transgenic, disease resistance and, 112
CAT gene, 268
Cathelicidins, 106
  distribution and spectrum of activity of, 109*t*
Cathepsin D, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Cathepsin K, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Cationic lipid-based DNA delivery, mechanism of, 163
Cattle production, genetic selection and, 313
CCL4, trout macrophage studies and, 212
CCV. *See* Channel catfish virus
cDNA, xii
  shrimp and subtracting highly redundant genes from, 22
  unmodified *vs.* modified, expected gene frequency distribution in, *25*
cDNA libraries, 94
  targeted, trait-relevant gene discovery and, 4–6
cDNA microarrays, cDNA clones and, 94
CD4+T cells, 126
CD8+T cells, 126
Cecropin, 108
Cell culture systems, choosing, 207
Cellular innate immunity, 123
CGP. *See* Atlantic Cod Genomics and Broodstock Development Project

cGRASP. *See* consortium for Genomics Research on All Salmonids Project
Channel catfish *(Ictalurus punctatus),* 3
  centromeric sequences identified in, 73
  cytokines as adjuvants for DNA vaccines and, 161
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
  viral DNA vaccines for, 156*t*
Channel catfish virus, DNA vaccines for, 156*t,* 158
Chemokine CCL4/MIP-1β, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Chemokines, as measures of immune responses, 126–127
Chemolithotrophic bacteria, ammonia oxidation and, 76
Chemotaxin, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Chicken, inbred lines of, 56
Chilling sensitivity, embryo cryopreservation and, 311
Chimera production, germ-line, confirming, 195–196
China (ancient), beginnings of culture of fish in, xi
Chinese shrimp *(Fenneropenaeus chinensis),* AMP sequences identified in, 107
Chinook salmon *(Onchorhynchus tshawytscha)*
  analyzing adaptive immune response of, and virulence of *V. anguillarum,* 129
  BAC libraries for, 75
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
  SD locus and, 75
  spawning channels used for, *131*
  viral DNA vaccines for, 155*t*
ChIP-on-chip arrays, 15
Chordin, DV patterning and, 184
Chromosome fragments, clonal lines and, 58
Chrysophin, 106
  distribution and spectrum of activity of, 109*t*
Chum salmon *(Onchorhynchus keta)*
  cryopreservation of, 312
  sex chromosomes and, 75
Ciba-Geigy, 283
CIHR. *See* Canadian Institutes for Health Research
cis-acting DNA regulatory elements, transgene expression and, 239
Clam *(Ruditapes decussatus),* AMP sequences identified in, 107
Clavanin, 106
  distribution and spectrum of activity of, 109*t*
Clavispirin, 106
  cytotoxic activity of, 110
  distribution and spectrum of activity of, 109*t*
Clodronate liposomes, 163
Clonal fishes
  advantages of clonal lines, 56–58
    ease of mitochondrial transfer, 57–58
    speed of generation, 56–57
    storage using cryopreserved sperm, 57
  clonal lines: repeatable experimental system
    crosses of clones to outbred animals, 59
    homozygous clones, 59
    hybrid clones, 59
  defined, 55
  disadvantages of clonal lines, 58
    chromosome fragments, 58
    limited sampling, 58
    poor fertility of females, 58
    poor survival rates, 58
  genetic analyses using clone crosses, 59–60
    genetic mapping, 59–60
    quantitative trait loci studies, 60
  harnessing the power of inbred lines, 55–56
  potential opportunities with, for aquaculture research, 65
  prospects and pitfalls of, in postgenomic era, 55–65
  utilization of DNA or RNA from clones, 60–61
    expression studies, 60
    identification of duplicate genes, 60–61
    whole genome sequencing, 61
Clonal lines
  advantages of, 56–58
    ease of mitochondrial transfer, 57–58
    speed of generation, 56–57
    storage using cryopreserved sperm, 57
  in aquaculture species, 56
  disadvantages of
    chromosome fragments, 58
    limited sampling, 58
    poor fertility of females, 58
    poor survival rates, 58
  heterozygous, 55
  homozygous, 55
Clonal line system, advantages and disadvantages of, 65
Clone crosses, genetic analyses and, 59–60
Clones
  case examples for utilizing in aquaculture-related research, 61–65
    alternative feeds in aquaculture, 61–62
    domestication, 63–64
    sexual dimorphism, 64–65
    temperature tolerance, 62–63
  crosses of, to outbred animals, 59
  homozygous, 59
  hybrid, 59
Cloning, of tilapia insulin gene, 282, 285
Cluster, 12
Cluster 3.0, 10*t,* 12
Clustering, 9
  of microarray data, 11–12
Cluster/Tree View, 10*t*
CMV-IEP.MCV1.4, DNA vaccine vector construction and, 160
CMV immediate early promoter/enhancer, 159
Coadapted gene complexes, 328
Cod *(Gadus morhua),* primary cell culture methods for head kidney from, 212
Coho salmon *(Onchorhynchus kisutch)*
  cosmid libraries for, 75
  identification of YY individual in, using GH-Y probe, *72*
  immune system in, 121
  number of ESTs in GenBank, 4*t*
  transgene localization in, 73–74
Colony stimulating factor 1 receptor
  microscopic changes in rainbow trout mononuclear phagocytes and, *210*
  primary cell culture methods for head kidney and, 211

Common carp *(Cyprinus carpio),* 3
β-actin promoter region in, 239
cytokines as adjuvants for DNA vaccines and, 161
maternal expression of sonic hedgehog in, 184
microarray platforms for, 7*t*
number of ESTs in GenBank, 4*t*
role of maternal mRNAs in oocyte quality and early embryonic development, 182*t*
viral DNA vaccines for, 155*t*
Competition, native habitats and, 327
Concatemerization, transgene, 238
Condition factor, defined, 297
Consequentialist ethics, 352
Consortium for Genomics Research on All Salmonids Project (cGRASP), 6, 84, 95
Continuous cell lines, advantages with, 207
Convergent evolution, 323
Cooling, controlled, sperm cryopreservation and, 308
Copy number, molecular level variation and, 324
Corn, inbred lines of, 56
Cosmid libraries, 75
Cotton congeners *(Gossypium barbadense),* 323
CPA. *See* Cryoprotectant agents
CPAs
embryo cryopreservation and, 311
oocyte cryopreservation and, 310
sperm extenders and, 308
Crabs, white spot syndrome virus in, 142
Crayfish, white spot syndrome virus in, 142
CRE-ERT fusion protein, Cre and, 245
Crustacean hyperglycemic hormone, RNAi-based experiments in Penaeid shrimp and, 30*t*
Crustaceans, white spot syndrome virus in, 142
Crustins, 106
RNAi-based experiments in Penaeid shrimp and, 31*t*
Cryobanking, reproductive technologies combined with, 313
Cryodamage, 306–307
Cryopreservation, 305–314, 347
applications, 313–314
broodstock management, 313
conservation of biodiversity, 314
germplasm banking and genetic selection programs, 313–314
preservation of biotechnological strains, 314
blastomere and primordial germ cell, 312–313
cell, basic principles of, 305–307
cryodamage, 306–307
slow, controlled freezing, 306
vitrification, 306
of clonal line sperm, 57
defined, 305
embryo, 310–312
gamete
oocyte, 309–310
sperm, 307–309
surrogate parent technology and, 229–230
Cryopreserved sperm, fish genetic material preserved in, 203–204
Cryoprotectant agents, 306
Cryoprotectent extender, sperm dilution in, *309*
Cryptobia salmositica, 121
Cryptocaryon irritans, piscidin 2 active against, 110
Cryptococcus, AMPs and inhibition of, 108
CSF1R. *See* Colony stimulating factor 1 receptor
CUB domain protein, RNAi-based experiments in Penaeid shrimp and, 30*t*
CXCR4, 108
Cyan fluorescent protein, 275
Cyclization of recombination (Cre)
activation of transgene expression by, *246*
cytotoxicity attributable to, 245–246
inducible systems and, 244–246
CyHV3 infections in koi carp, vaccine against, 153
CYP1a1, 272
Cyprinidae, zebrafish within, 268
Cypriniformes
examples of species with genomic resources and number of ESTs in GenBank, 4*t*
selected microarray platforms, overview of, 7*t*
Cytochrome P450 family, 272
Cytokines
as adjuvants for DNA vaccines, 160–161
coadministration of, with vaccines, 112
live attenuated bacteria and, 128
as measures of immune responses, 126–127
Cytoplasmic microinjection, transgenic fish creation by, 323

D
Dalhousie University, Aquatron at, 285, 286
Database for Annotation, Visualization and Integrated Discovery. *See* DAVID
Data visualization and processing, 10–11
DAVID, 10*t,* 12
Days postinfection, 87
DEAD protein family, 180
Defensin, 106
distribution and spectrum of activity of, 109*t*
Deontological ethics, 352
Designer promoters, in transgenic fish, 275
Diabetes mellitus
fish islets and treatment of, 289
insulin-dependent, 281
Dicer 1, RNAi-based experiments in Penaeid shrimp and, 31*t*
Dicynthaurin, 106
Differential expression
cloning studies in Penaeid shrimp, 26*t*–28*t*
understanding gene function and, 22, 25
Dilemma, defined, within context of ethics, 346
Dimethylsulfoxide
as cryoprotectant, 306
embryo cryopreservation and, 311
Direct feminization, description of, 44–45
Disease
control of, using AMPs, 111–113
direct administration, 111–112
DNA vaccines, 112
genetic selection for enhancement of disease response, 112
transgenic expression, 112–113
vaccine adjuvants, 112
control of in aquaculture, current approaches to, 110–111
ecological effects of, 327
Disease diagnostics, xii

Disease resistance, transgenic technology and, 267
Disease responses, genetic selection for enhancement of, 112
Diversity of fish, surrogate parent technology and, 229–230
DMEM
  cell culture methods for satellite cells and, 214, 215
  containing 10% FBS, differentiation of rainbow trout muscle satellite cells cultured at 18° C with, *215*
  primary cell culture methods for head kidney and, 208
DMSO. *See* Dimethylsulfoxide
DNA
  clones and utilization of, 60–61
  sperm cryopreservation and integrity of, 308
DNA:DNA hybridization, *A. salmonicida* studies and, 91
DNA microarrays, xii, 3, 13
DNA regulatory elements, identification of, 239, 241
DNA sequencing technologies, xii
DNA vaccines, 112, 113
  for aquaculture animals, 155*t*–156*t*
  autogenous vaccines *vs.,* 98
  candidates for, 156–158
  components in, 154
  conclusions and future directions for, 167
  cytokines as adjuvants for, 160–161
  delivery methods for, 161–163
    advantages and disadvantages with, 162*t*
    formulating plasmid DNA, 161–162
    gene transfer inefficiency, 161
    intradermal and intramuscular injection, 161
    liposomes, 162–163
    mechanism of cationic lipid-based DNA delivery, 163
    use of liposome-formulations in fish, 163
  development of
    criteria for, 154
    research targets for, *157*
  formulations, 158–160
    vector construction, 158–160
  overview of, 153–154
  public opinion, 166–167
  regulatory constraints, 165–166
    persistence and distribution of plasmid DNA, 166
  response of immunized host and, 164–165
    injection-site EAVR, 164
    late antiviral responses, 165
    systemic antiviral responses, 165
    systemic EAVR, 164–165
  for viral diseases of farmed fish and shellfish, 153–167
DNA viruses, DNA vaccines for, 158
dnd, RT-PCR analysis of, 201, *202*
Domestication process, clonal lines and, 63–64
Domestic selection and divergence, influence of, 325
Dosage effects, molecular level variation and, 324
Double (flpA-tapA) mutant, bioinformatic analysis of *A. salmonicida* and, 92
"Double haploid" offspring, clonal lines and, 56
Double muscled cattle, myostatin deficiency in, 291
Doyle, Roger, 286
DPI. *See* Days postinfection
Drosophila, 224
  Gal4-UAS system and, 242
  inbred lines and, 56
  study of maternally expressed genes in, 180
  transposons and, 236
DsRed. *See* Red fluorescent protein
dsRNA
  injections of, in shrimp, 29, 34
  RNAi-based experiments in Penaied shrimp and, 33*t*
Dulbecco's Modified Eagle Medium. *See* DMEM
Duplicate genes, clonal lines and identification of, 60–61
Duty, ethical dilemma and, 346–347
DV patterning, Japanese killifish and, 184

E

Early antiviral responses (EAVRs), 164
  injection-site, 164
  systemic, 164–165
Ecology, species invasions and effects on, *326*
Economic development, aquaculture industry and, 348
Edmonton Protocol, 281
Edwardsiella ictaluri
  genomic sequencing of, 90*t*
  studying virulence genes of, 92
  transgenic catfish and resistance to, 112
Edwardsiella tarda, genomic sequencing of, 90*t*
Eel *(Anguilla anguilla),* primary cell culture methods for head kidney from, 212
Eggs
  fertilization of, thawed sperm and, 308
  transdifferentiation of spermatogonia, from adult fish into, 227–228
Egg transcriptome, 177–187
  introduction on, 178
  as maternal legacy to embryo, 177–187
Egypt (ancient), beginnings of culture of fish in, xi
Electrophile response elements, response of, to environmental pollutants, 276*t*
Embryo cell culture, derived from blastula showing ES-like morphology with aggregates of embryo cells, *194*
Embryo cryopreservation, 310–312
Embryogenesis, analyzing role of maternal mRNA during, 180
Embryogenesis signaling, transforming growth factor beta superfamily of secreted cytokines and, 183–184
Embryological studies, morpholinos and, 88
Embryonic development, early, maternal factors and driving of, 181
Embryonic stem (ES) cell cultures, mouse, genetic alterations introduced into germ line with, 193
Embryos
  early, mRNA in before mid-blastula transition, 179–181
  early, transcriptomic analysis of, 178–179
  egg transcriptome as maternal legacy to, 177–187
  newly hatched, immunologic immaturity of, 224–225
  newly hatched, microinjection of germ cells into peritoneal cavity of, *226*

Embryos (*Continued*)
prehatch, hybrids between trout and salmon, *226*
Empirical premises, ethical arguments and, 350
Encapsulation, ideal islet tissue for, 288
Endocrine sex reversal, Atlantic halibut and, 45–46
Endosomal escape, efficient gene delivery and, 163
Enhanced growth rate, transgenic technology and, 267
Enhancers
in genes, *240*
transgene expression and, 239, 241
Entrez human gene symbols, 12
Enveloping layer, Irf6 and differentiation of, in zebrafish embryos, 184
Environment, aquaculture and, 349, 351
Environmental ethics, pluralistic expression of, 351
Environmental monitoring, 347
Environmental pollutants, summary of response element responses to, 276*t*
ERE. *See* Estrogen response element
Ergosan, 130
ER LBD, modification of, 245
ES cell pluripotency, ultimate test of, 195
ES cells, transgenic tilapia and, 287
Escherichia coli
AMPs and, 113
DNA-derived VP2 and, 157
live attenuated bacteria and, 128
tetracycline regulated system and, 244
EST. *See* Expressed sequence tags
EST collections. *See* Expressed sequence tag (EST) collections
EST databases. *See* Expressed sequence tag (EST) databases
Estrogen, direct feminization and, 45
Estrogenic compounds
biomonitoring transgenic fish systems and, 270*t*
models of biomonitoring fish and, 269, 271
Estrogen receptors, DNA vaccine vector construction and, 159
Estrogen response element, response of, to environmental pollutants, 276*t*
EST surveys, antimicrobial peptides and, 107
Ethical arguments, normative components and, 350
Ethical decision-making, 346, 352
Ethical dilemma, defined, 345
Ethical dimensions, facts and substance of, 345
Ethics, 352
of aquaculture in biotechnology century, 345–352
from facts to ethical issues, 350–352
factual content, for and against, 350*t*
features of a controversy, 348–350
modern aquaculture as a focal point, 347–348
role of, 346
Ethylene glycol, embryo cryopreservation and, 311
E2
LOEC of, for hemizygous transgenic fish, 269
Tg(mvtg1:gfp) transgenic medaka and induction of GFP expression by, *271*
Eukaryotic gene expression, control of, 239
European flounder
gene expression-based toxicogenomics approach applied to, 277
transcriptional response of liver of, 95
European sea bass *(Dicentrarchus labrax)*
microarray platforms for, 7*t*
number of ESTs in GenBank, 4*t*
Evolutionary processes, at transgene loci, 324
Exons, in genes, *240*
Exotic habitats, *326*
Exploitative competition, ecological effects of, 327
Expressed sequence tag (EST) collections
gene discovery in shrimp and, 21–22
of selected aquaculture fish species, 4*t*
Expressed sequence tag (EST) databases, 3
Expressed sequence tags, 83
defined, 21
Expression studies, utilization of DNA or RNA from clones and, 60–61
Extenders
cryopreservation and, 306
sperm cryopreservation and formulation of, 307–308
Extinction issues, germ cell transplantation in fish and, 229–230

F
FACS. *See* Fluorescence-activated cell sorting
Facts, substance of ethical dilemmas and, 345
Factual claims, verifying accuracy of, 350
Fadrozole, masculinizing fish with, 45
False discovery rates, 11
Farmed fish. *See* DNA vaccines, for viral diseases of farmed fish and shellfish
Farmed populations
interbreeding of wild populations and, 327
selective pressures on, 323
Fathead minnow *(Pimephales promelas)*
frog virus 3 and, 88
gene expression-based toxicogenomics approach applied to, 277
FDA. *See* Food and Drug Administration
FDRs. *See* False discovery rates
Feed conversion efficiency, 347
Female gametes, cryopreservation of, 309–310
Females, clonal lines and poor fertility of, 58
Female stocks, of Atlantic halibut, production of, 44–46
Femtosecond lasers, embryo cryopreservation and, 312
Fenneropenaeus chinensis
differential expression cloning studies in, 28*t*
genomic research by EST analysis for, 22
microarrays with gene contents and, 25
Fertilization, *309*
Fertilization rates, sperm cryopreservation and, 308
Fiber (muscle) diameter distribution, from transgenic and control fish, *300*

Finfish
adaptive immunity in, 121–132
antigen presentation in adaptive immune response, 125–126
Atlantic cod 20K oligonucleotide microarray, 9
chromosome set manipulated, characterization of interspecific hybrids and, 75
cytokines and chemokines as measures of immune responses, 126–127
future of biotechnology and immunology in aquaculture, 131–132
future recommendations for microarray research, 13–15
genomic tools for understanding molecular basis of production-relevant traits in, 3–15
immune system as whole integrative defence mechanism, 121–125
humoral innate immunity, 122–123
main features of fish adaptive immune system, 123–125
main features of fish innate immune system, 122
immunostimulants, 129–130
improving broodstock and breeding practices, 130–131
knowledge of fish immunity and what it means for vaccines, 127–129
adjuvants, 128
live attenuated bacteria as carriers, 128–129
method of delivery, 128
MH receptors, 125
MH sequences and their applications, 126
microarray data analysis tools, 9–13
clustering, 11–12
data visualization and processing, 10–11
functional annotation of gene lists, 12–13
statistical analysis of gene expression differences, 11
microarray technology applied to aquaculture and research on, 6, 9–13
monitoring of health status, 130
qPCR, 129
selected microarray platforms, overview of, 7*t*–8*t*
targeted trait-relevant gene discovery, 4–6
FISH. *See* Fluorescence in situ hybridization
Fish
antifreeze proteins isolated from, 254*t*
aquaculture and welfare of, 349
contribution of antifreeze proteins to freeze protection of, *256*
control and transgenic, muscle sections (representative) from, *300*
extinction dangers facing, 314
germ cell transplantation in, 223–230
monitoring health status of, 130
mononuclear phagocytes in, 208
myostatin and, 293–294
pathogens identified in, 76–77
Fish antifreeze protein genes, xi
Fish cell cultures, genetic manipulation of, 196
Fish egg quality, defined, 177
Fish farms, utilitarian principle and, 351
Fish health, 83–98
bacterial pathogens-*A. salmonicida,* 89, 91–93
genomic variability and subspecies identification, 91–92
proteomics, 92–93
genomics and proteomics applied to vaccine development, 97–98
genomics applied to studies of, 83–84
host-pathogen interactions, 93–97
host metabolomics, 96–97
host proteomics, 96
metabolomics applied to studies of, 84–85
proteomics applied to studies of, 84
studies of pathogen biology, 85–93
viral pathogens-Iridoviridae, 85–93
functional information from sequence data, 86–87
genomic studies, 85–86
molecular diagnostic test development, 86
proteomics, 87–89
Fish meal
alternative protein for, 62
as protein source in aquaculture, sustainability issues and, 61
Fish promoters, DNA vaccine vector construction and, 159–160
Fish skeletal muscle cells, functional characterization of and their application in aquaculture, 216–219
Fish sperm, characteristics of, 307
Fish stem cell technology, application of, to aquaculture and marine biotechnology, 193–204
5S rDNA, locations of, in fish species, 72–73
Flatfish pleuronectin promoters, DNA vaccine vector construction and, 160
Flatfish species, sexually dimorphic size variation in, 44
Flavobacterium psychrophilum, genomic sequencing of, 90*t*
fli1 promoter, 242
Floxed Transgene X construct, transgenic fish with cell-specific Cre expression and, *246*
FLP pili (flpA), bioinformatic analysis of *A. salmonicida* and, 92
Fluorescence-activated cell sorting, 199
Fluorescence in situ hybridization
application of, in aquaculture-related research, 71–77
assignment of genetic linkage groups to specific chromosomes, 75–76
characterization of interspecific hybrids and chromosome set manipulated finfish, 75
future applications of, 77
identification and characterization of sex chromosomes, 74–75
identification of pathogens in cultured shellfish, fish, and wastewater generated by aquaculture, 76–77
localization of repetitive sequences, transposons, and transgenes, 71–74
use of, 71
Fluorescent proteins, multicolor transgenic biomonitoring fish and, 275–276
Fluorescent transgenic embryos/larvae, toxicological screening and use of, *273,* 273–274

Follistatin, *292*
myostatin bound by, 293
overexpression of
quadrupling of mice muscle mass and, 293
"six pack" phenotype in P1 juvenile transgenic fish and, 296, *298*
in transgenic rainbow trout, 291
in trout, muscle growth and, 296
Follistatin coding sequence, rainbow trout, *mylc* expression and, *295*
Follistatin construct
DNA, production statistics for rainbow trout eggs injected with, 296*t*
rainbow trout and, 294–295
Follistatin overexpressing transgenic trout, transgenic F1 offspring and future of, 300
Follistatin transcript levels, in 50 ng of RNA from transgenic and control RBT, *297*
Food and Drug Administration, xii
DNA vaccine safety and, 165–166
Food preservation, AMPs and, 113
Food production, population growth and, 131
Food security, aquaculture's role in, 348
Forkhead Box H1 protein, 184
Freeze protection of fish, antifreeze proteins and, *256*
Freeze resistance, transgenic technology and, 267
Freeze-resistant fish, producing for aquaculture, summary, 263
Freeze-resistant salmon, future directions in production of, 260–263
Freezing
adverse effects of, 305–306
slow and controlled, 306
of sperm, 308, *309*
French straws, sperm loading in, *309*
Frog virus 3 (FV3), 88, 108
Fugu *(Takifugu rubripes)*
cytokines as adjuvants for DNA vaccines and, 161
genome sequences available for, xii
Functional annotation of gene lists, 12–13
Functional genomics, 4–6, 13–15, 83
Funding, for breeding programs, 286
Fusarium oxysporum, AMPs and inhibition of, 108
G
GA, 13
Gadiformes, selected microarray platforms, overview of, 7*t*
Galectin, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Galectin-like protein
EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Gal4 enhancer trap lines
efficient generation of, 244
in neural circuits of zebrafish, 243
GAl4 protein, 242, 243
Gal4-UAS, 242–244
Gal4-UAS binary system, for tissue-specific expression of transgenes, *243*
Gal4-VP16 protein, 242
Gal4-VP16 TF, 244
Gamete cryopreservation, 307–310
oocyte cryopreservation, 309–310
sperm cryopreservation, 307–309
Gametogenesis, shrimp aquaculture and, 25
Gastrulation, maternal factors involved in, 184–185
GATA-2 gene, 241
GDF. *See* Growth and differentiation factors
GDF-11, 293
GenBank, number of ESTs in, 4–6, 4*t*
Gene carriers, ideal, properties for, 161
Gene constructs, uses for, 235
Gene delivery methods, DNA vaccines and advantages/disadvantages of, 162*t*
Gene discovery efforts, cDNA libraries and, 4–6
Gene expression, 21
differential expression and, 22
Gene expression analysis, 11
Gene expression arrays, 15
Gene Expression Omnibus (GEO) database, 6, 186
Gene knockdowns, RNA interference (RNAi) and, 288
Gene knock-down studies, for maternal factors involved in germline establishment, 181, 183
Gene lists, functional annotation of, 12–13
Gene microinjection technique, 284
Gene Ontology (GO) annotation, 13
Generation time, Atlantic halibut and impact of, 50
Genes
in fish, paucity of knowledge about in 1980s, xii
structure of, with exons, promoter region, and enhancers, *240*
Gene Set Analysis, 9, 10*t*
Gene set enrichment analysis, 10*t,* 12
Genesis, 9, 10*t,* 12
GeneSpring GX, 9
Genetic linkage groups, FISH and assignment of, to specific chromosomes, 75–76
Genetic maps/mapping, 21
homozygosity of clonal lines and, 59–60
for shrimp species, 35–36
Genetic markers, shrimp and development of, for breeding purposes, 35
Genetic programming optimization of neural networks, 36
Genetic selection programs, cryopreservation and, 313–314
Genetic variability, halibut pedigree studies and, 46–46
Gene transfer inefficiency, DNA vaccines and, 161
GenMAPP, 10*t,* 12
Genome Analyzer, 35
Genome Canada, 132
Genome mapping, comparative, FISH and, 77
Genome sequencing, clonal lines and, 61
Genomic DNA libraries, xii
Genomics
defined, 83
fish health studies and application of, 83–84
vaccine development and, 97–98
Genomic Southern blotting, xii
Genomics studies, of shrimp, analytical challenges with, 36–37
"Genomics toolboxes," aquaculture research and, 3
Genomic studies, viral pathogens-Iridoviridae and, 85–86
GenePix, 9
GEO, 13
Geodia barretii, 77

Germ cell transplantation in fish, 223–230, 347
  germ cell migration to gonads, 225–226
  immunological immaturity of newly hatched embryos, 224–225
  principle for production of eggs and sperm of different species in surrogate parents by, *224*
  prospective view of, 229–230
  sterile triploid fish with normal somatic cells, 228–229
  transdifferentiation of spermatogonia from adult fish into eggs, 227–228
  vasa gene in specifically expressed germ cells, 223–224
Germ-line chimera production, from zebrafish PGC cultures, 202–203
Germ-line chimerism, confirming, 195–196
Germline establishment, gene knock-down studies for maternal factors involved in, 181, 183
Germ plasm, defined, 180
Germplasm banking, cryopreservation and, 313–314
Germplasm cryobanks, conservation of biodiversity and, 314
G418, 200
GFP. *See* Green fluorescent protein
GFP transgenic fish, GFP protein synthesis in muscle tissue of, *297*
GH. *See* Growth hormone
GH-enhanced phenotypes, growth-selected phenotypes *vs.,* for salmonid fishes, 328, 334–335
GH-insulin-like growth factor I system, 328
GH-Y probe, identifying YY coho salmon and, 75
Gill epithelial cells, rapid turnover rate of, 257–258
Gill filament, cellular distribution of AFP-producing cells in, *258*
Gill lamellae epithelia, intracellular AFP in, 257
Gilt-head seabream *(Sparus aurata)*
  ES-like cell lines established for, 195
  microarray platforms for, 7*t*
  number of ESTs in GenBank, 4*t*
  primary cell culture methods for head kidney from, 212
  sperm cryopreservation studies for, 307
Glassy solid state, vitrification and, 306
Global food supply, in 21st century, biotechnologies and, 336
Global seafood supply, aquaculture industry and, 348
GloFish, 267
Glucans, enhanced immune responses and, 129
Glucocorticoid response element, response of, to environmental pollutants, 276*t*
GLUT4
  endogenous, subcellular localization of, in trout muscle cells, *218*
  functional characterization of fish skeletal muscle cells and, 216, 217, 218
Glyceraldehyde 3-phosphate dehydrogenase, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Glycerol, as cryoprotectant, 306
Goldfish *(Carassius auratus),* ActRIIB protein injection and increased size of, 294
Gonadal differentiation, Atlantic halibut and, 45–46
Gonad-inhibiting hormone, RNAi-based experiments in Penaeid shrimp and, 30*t*
Gonads, germ cells seeking out and migrating to, 225–226
gp64, RNAi-based experiments in Penaied shrimp and, 32*t*
gp116, RNAi-based experiments in Penaeid shrimp and, 32*t*
GPNN. *See* Genetic programming optimization of neural networks
Granulocyte-macrophage colony stimulating factor, 160
GRE. *See* Glucocorticoid response element
Great scallop (*Pecten maximus),* type of karyotype in, and location for major and minor rDNA loci, 73
Green fluorescent protein, 159, 195, 267
Green fluorescent protein (GFP) genes, germ cell transplantation and, 224, *225*
Green revolution, 348
Green sea urchin *(Strongylocentrotus droebachiensis),* cysteine-rich AMP from, 106–107
Growth and differentiation factors, 292
Growth enhancement, 347
Growth hormone
  DNA vaccine vector construction and, 159
  skeletal muscle and effects of, 219
Growth rate, enhanced, identifying genes contributing to, examples, *14*
Growth-selected phenotypes, GH-enhanced phenotypes *vs.,* for salmonid fishes, 328, 334–335
GSA, 12
GSEA, 12
GS FLX Titanium, 13
Gynogenesis
  Atlantic halibut and, 45
  clonal lines and, 56
  homozygous clones and, 59
Gynogenetic fish, producing, 45

H
Habitat restoration, extinction issues and, 229–230
Hag, 196
Halibut aquaculture, establishing feasibility of, 43
Halibut genetic linkage map, 47, 49
Halibut *(Hippoglossus hippoglossus)*
  antifreeze proteins and, *256*
  freezing point of (without AF(G)P), 255
  long generation time of, 44, 49
  pedigree analysis and production of, 46–47
Halocidin
  antifungal activity of, 108, 110
  distribution and spectrum of activity of, 109*t*
"Happiness of the greatest number" principle, aquaculture and, 351
Hatcheries
  halibut broodstock selection and, 50–51
  pedigree analysis and, 46–47
Head kidney, primary cell culture methods for, 208–212
Head kidney cells
  from rainbow trout, dissection and isolation of, *209*
  studies and use of, 208
Health biomarkers for fish, development of, 97

Health status of fish, monitoring, 130
Heat shock, inducible systems and, 246–247
Heat shock elements, 247
Heat-shock-like protein, DNA vaccine vector construction and, 159
Heat shock protein 70, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Heat shock proteins, 247, 271
Heat shock response element, response of, to environmental pollutants, 276*t*
Heat stress resistance, identifying genes contributing to, examples, *14*
Heavy metals
  biomonitoring transgenic fish systems and, 270*t*
  models of biomonitoring fish and, 271–272
Helicase-coding gene, RNAi-based experiments in Penaeid shrimp and, 32*t*
Hemocyanin, RNAi-based experiments in Penaeid shrimp and, 30*t*
Hepcidin, 106
  distribution and spectrum of activity of, 109*t*
Herring sperm, first report on cryopreservation of, 307
Heterochromatin formulation, concatener expression and, 238
Heterologous regulatory sequences, 241–242
Heterozygous clonal lines, 55
High-throughput studies, of differential expression and gene discovery in shrimp, 22, 25, 29
Hirame rhabdovirus (HIRRV), viral DNA vaccines against, 155*t*
Histidine-rich clavanins, 106
Histones, 106
HIV-1, mussel defensins active against, 108
Homologous regulatory sequences, 241
Homozygosity, of inbred lines, 55
Homozygous clonal lines, 55
Homozygous clones, 59
Homozygous females, poor fertility of, 58
Host defense peptides, 105. *See also* Antimicrobial peptides
Host defenses, viral envelope proteins and, 147
Host-pathogen interactions, 93–97
  fish health and
    host metabolomics, 96–97
    host proteomics, 96
  studies, RNAi-based experiments in Penaeid shrimp and, 31*t*
Host signaling pathways, regulating, viruses and, 88
Hox gene promoters, 242
HRE. *See* Hypoxia response element
HSEs. *See* Heat shock elements
HSP70, 271, 272
hsp70 promoter region, testing of, in heterologous scenarios, 242
HSPs. *See* Heat shock proteins
Human alpha defensin I, 108
Human health, aquaculture and, 349
Humoral immune response, variation in, among fish, 121
Humoral innate immunity, 122–123
Hybrid clones, 59
Hybridization, *326*
Hyperplasia of muscle fibers, in transgenic fish, 298–299
Hypoxia response element, response of, to environmental pollutants, 276*t*

I
ICAT. *See* Isotope-coded affinity tag
ICAT identification, of differentially expressed shrimp proteins during WSSV infection, 145
Ice, AFP binding to, *255*
Ice conditions
  in aquaculture sea cage operation during a severe winter, *259*
  at sea cage sites, example, 258
Ice nucleation, 305
Ichabod phenotype, 183
Ichthyophthirius multifiliis, piscidin 2 active against, 110
Ictalurid herpes virus 1, 158
Ictalurids, cell lines from ATCC available for, 207
I-FABP gene, reporter gene expression to zebrafish intestine and, 242
IGF-I, trout skeletal muscle cells and, 216
IHNV. *See* Infectious hematopoietic necrosis virus
Illumina, 15
Imagene, 9
Immune gene discovery in fish, vaccines and, 98
Immune immaturity, of newly hatched embryos, transplanted cells and, 224–225
Immune responses, shrimp aquaculture and, 25
Immune system
  cellular innate immunity, 123
  fish adaptive, main features of, 123–125
  humoral innate immunity, 122–123
  main features of, in fish, 122
  as whole integrative defence mechanism, 121–125
Immunization process, description of, 160
Immunology, future of, in aquaculture, 131–132
Immunostimulants, 110, 129–130
Inbred lines, clonal fishes: harnessing power of, 55–56
Incentives, ethics and, 346
Incubation, primary cell culture methods for head kidney and, 209–210, 212
Indinavir, inhibition of trout GLUT4 *vs.* mammalian GLUT4 and, 217–218
Indirect effects, abiotic and biotic, *326*
Indirect feminization, description of, 45
Inducible gene expression systems, 244–247
  cyclization of recombination, 244–246
  heat shock, 246–247
  tetracycline regulated system, 244
Industrial aquaculture, environment and, 351
Infectious hematopoietic necrosis virus, 154
  antiviral activity of AMPs and, 108
  systemic antiviral responses and, 165
  vaccine efficacy trials reported for, 156
  viral DNA vaccines against, 155*t,* 156*t*
Infectious pancreatic necrosis virus, 153
  antiviral activity of AMPs and, 108
  DNA vaccine trials for, 157
  viral DNA vaccines against, 155*t*
Infectious salmon anemia virus, vaccinations against, 155*t,* 157–158

Infectious spleen and kidney necrosis iridovirus, genus, accession number, and references, 85*t*
Insulator sequences, 239
Insulin
  DNA vaccine vector construction and, 159
  tilapia *vs.* human, differences in, 282, 288
  trout skeletal muscle cells and, 216, 217, 218
Insulin-like growth factor II, DNA vaccine vector construction and, 159
Insulin-stimulated 2-deoxyglucose uptake, in trout muscle cells, time course of, *217*
Intentional selection, in aquaculture broodstocks, consequences of, 324
Interbreeding, 325–326
  native habitats and, 327–328
Interference competition, ecological effects of, 327
Interferon regulatory factor 1, *5*
Interferon regulatory factor 6 (Irf6), gastrulation and, 184
Interleukin-1, 160, 212
Interleukin-2, 160
Interleukin-8, 161
  EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Interleukin-18, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Interleukin 1 receptor, type II, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Interspecific hybrids, characterization of, 75
Introgression, native habitats and, 327–328
IPNV. *See* Infectious pancreatic necrosis virus
IRF1. *See* Interferon regulatory factor 1
Iridoviridae, 142
  host-pathogen interactions and, 94
  viral pathogens, 85–89
    functional information from sequence data, 86–87
    genomic studies, 85–86
    molecular diagnostic test development, 86
    proteomics, 87–89
  viruses within, for which there are completed genomic sequences, 85*t*
Iridoviruses, defined, 142
ISAV. *See* Infectious salmon anemia virus
ISKNV. *See* Infectious spleen and kidney necrosis iridovirus
ISKNV genome, bioinformatic analysis of, 88–89
islet1 regulatory region, 242
Islets of Langerhans, replacing, diabetes treatment and, 281
Isobaric tags for relative and absolute quantification. *See* iTRAQ
Isoelectric focusing, 2D PAGE and, 84
Isotope-coded affinity tag, 143
iTRAQ, 87, 143
  analysis of SGIV infected grouper embryonic cells, 145, 147
  for viral protein localization in WSSV, 145
IWK Children's Hospital (Halifax), 282

J
Japanese flounder *(Paralichthys olivaceus),* 86
  length of time CAT protein expressed in, 159
  microarray platforms for, 7*t*
  viral DNA vaccines for, 155*t,* 156*t*
Japanese killifish *(O. latipes),* DV patterning and, 184
Japanese Medaka, establishment of, as vertebrate model, xii
Java TreeView, 10*t*
JDFI. *See* Juvenile Diabetes Foundation International
JDRF. *See* Juvenile Diabetes Research Foundation
Juvenile Diabetes Foundation International, 283
Juvenile Diabetes Research Foundation, 284
Juvenile-onset diabetes, 281

K
Kanamycin, 166
katA, 92
KEGG, 12, 13
kita, RT-PCR analysis of, *202*
kitb, RT-PCR analysis of, *202*
Kitlga, recombinant, effect on growth of zebrafish PGCs, 198–199, *199*
Kitlgb, recombinant, effect on growth of zebrafish PGCs, 198–199, *199*
k-means clustering, 11–12
Knockout technology, future improvements to transgenic tilapia and, 287
Koi carp
  vaccine against CyHV3 infections in, 153
  viral DNA vaccines for, 155*t*
Kokanee salmon *(Onchorhynchus nerka),* viral DNA vaccines for, 155*t*
Kyoto Encyclopedia of Genes and Genomes. *See* KEGG

L
Lactococcus garvieae
  genomic sequencing of, 90*t*
  studying virulence genes of, 92
LacZ gene, 268
Laminin, cell culture methods for satellite cells and, 215
LAMP, detection of fish/shellfish pathogens and, 86
Land use, aquaculture and impact on, 349
Large insert size genomic libraries, for shrimp species, 36
Large yellow croaker iridovirus, genus, accession number, and references, 85*t*
Late antiviral responses (LAVRs), 165
LBD. *See* Ligand-binding domain
LCDV. *See* Lymphocystis disease virus
Leishmania major, mussel defensins and fragments active against, 110
Leukemia inhibitory factor, human and mouse, zebrafish ES cell cultures and, 195
Leventhal, Joe, 284
LIF. *See* Leukemia inhibitory factor
Ligand-binding domain, 245
Limma, 10, 10*t,* 11
Linkage mapping, Atlantic halibut and, 47, 49
Lipofectin, 163
Liposome-formulations, 163
Liposomes
  defined, 162
  gen delivery and, 162–163
  various uses for, 163

Listonella anguillarum, first vaccine for, 153
LITAF, viral replication and, 88
Litopenaeus setiferus
differential expression cloning studies in, 26*t,* 27*t*
genomic research by EST analysis for, 22
Litopenaeus stylirostris
differential expression cloning studies in, 26*t*
microarrays with gene contents and, 25
Live attenuated bacteria, as carriers, 128–129
Live attenuated vaccines, xi, 153
Liver-type AFP
skin-type AFP *vs.,* 257
winter flounder and production/secretion of, 256
Liver type 1 AFP, improving freeze resistance of rainbow trout and, *261*
Livestock husbandry, intensive, 347
Loaches, clonal lines of, 55
Localization of repetitive sequences, FISH and, 71–74
LOEC. *See* Lowest-observed-effects concentration
London Life Insurance Company, 282
Long terminal repeat, of zebrafish, DNA vaccine vector construction and, 160
Lowest-observed-effects concentration, of E2 for hemizygous transgenic fish, 269
Low sea water temperatures, danger posed to fish by, 253
Low temperature limitations, to sea cage aquaculture, 258–259
LPS, studies using trout macrophages and, 212, 214
LPS-stimulated macrophage cDNA libraries, comparison of selected EST frequencies between 12 and 30 hour, 213*t*
LTR. *See* Long terminal repeat
luciferase, 268
LYCIV. *See* Large yellow croaker iridovirus
Lymphocystis disease virus
DNA vaccines for, 158
genus, accession number, and references, 85*t*
viral DNA vaccines against, 156*t*
Lymphocystivirus, full genome sequences available for, 85

M
Mackerel *(Scomber japonicus),* germ cell transplantation and, 223
MacroGard, 130
Macrophage receptor with collagenous structure
microscopic changes in rainbow trout mononuclear phagocytes and, *210*
primary cell culture methods for head kidney and, 211
Macrophages, differentiated, obtaining, 208
Maine hatchery, halibut broodstock pedigree analysis, 48*t*
Major histocompatibility (MH) genes, 123
Male fingerlings, tilapia growers and preference for, 74
Mammalian islets, tilapia islets and, 282
Mammalian promoter regions, 242
Mammals, inhibition of myostatin in, 293
MARCO. *See* Macrophage receptor with collagenous structure
Marine animals, protein needed in human nutrition and, 348
Marine biotechnology, fish stem cell technology applied in, 193, 203–204
Marine Genomics website, 22
Marine invertebrates, diversity of AMPs from, 106–107
Marker-assisted broodstock development, 322
Marker-assisted selection
Atlantic halibut and, 50–51
of finfish with superior production traits, 14, *14*
of superior broodstock, 3
Marker-replacement mutagenesis, 91–92
Marray, 10*t*
Marsupenaeus japonicus
differential expression cloning studies in, 26*t,* 28*t*
genomic research by EST analysis for, 22
microarrays with gene contents and, 25
MAS. *See* Marker-assisted selection
Masculinization of females, 45
Mass selection, aquaculture and, 50
Mass spectrometry, 141
Masu salmon *(Onchorhynchus masou)*
germ cell transplantation in, 223
male, rainbow trout embryos obtained from, after transplanting trout primordial germ cells, *226*
rainbow trout spermatogonia transplanted into, 227–228
testis triploid, cross sections of, *229*
triploid, germ cell transplantation and, 228–229
triploid, ovaries from into which rainbow trout spermatogonia had been transplanted, *230*
Masu salmon parents, female, rainbow trout embryos obtained from, *228*
Maternal-embryo-transition, zygotic transcription and, 177
Maternal mRNAs
degradation patterns of, throughout embryonic development, 177, *178*
detecting, methods for, 180
major role played by, 187
in oocyte, 177
role of, in oocyte quality and early embryonic development, 181, 182*t,* 183–186
gastrulation and, 184–185
gene knock-down studies, germline establishment and, 181, 183
neurogenesis and, 185
pattern formation and, 183–184
somitogenesis and, 185
study of, in unfertilized eggs of varying quality, 185
transcriptomic analysis of oocyte quality in fish, 185–186
for which differential abundance evidenced using QPCR in eggs of varying quality, 187*t*
Matrix metalloproteinase 9
EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
MBT. *See* Mid-blastula transition
Mcl-1, *5*
MDHT. *See* 17alpha-methyldihydrotestosterone
Medaka ES cells, establishing culture conditions for, 195
Medaka *(Oryzias latipes)*
gene expression-based toxicogenomics approach applied to, 277
genome sequences available for, xii

heat shock response tested in, 247
MBT in, 180
oocyte cryopreservation studies and, 310
role of maternal mRNAs in oocyte quality and early embryonic development, 182*t*
as sentinels in aquatic technology, 268
successful transgene integration in, 238
Tg(mvtg1:gfp) transgenic, induction of GFP expression by E2, *271*
transgene expression and transmission in, 236
vasa RNA expression in, 183
vtg mRNAs inducible in, 269
Medium-throughput studies, of differential expression and gene discovery in shrimp, 22, 25, 29
Megalocytivirus, full genome sequences available for, 85
Melittin, cytotoxic activity of, 110
Membrane protectants, sperm extenders and, 308
Memory immune response, 124
Messenger RNA (mRNA)
maternal, methods for detection of, 180
in oocyte and early embryo before mid-blastula transition, 179–181
MET. *See* Maternal-embryo-transition
Metabolite profiles, determination of, 84
Metabolome, 84
Metabolomics
defined, 84
fish health studies and application of, 84–85
host, fish health and, 96–97
Metallothionein A, DNA vaccine vector construction and, 159
Metallothionein B, DNA vaccine vector construction and, 159
Metal response element, response of, to environmental pollutants, 276*t*
Methanol, embryo cryopreservation and, 311
MGD-1,2, distribution and spectrum of activity of, 109*t*
MH complex, 125
MH receptors, types of, 125
MH sequences, applications of, 126
MIAME. *See* Minimum Information About a Microarray Experiment
Mice, inbred lines of, 56
Microarray-based comparative genomic hybridization, *A. salmonicida* studies and, 91
Microarray data analysis tools, 9–13
clustering, 11–12
data visualization and processing, 10–11
functional annotation of gene lists, 12–13
noncommercial software, 9, 10*t*
statistical analysis of gene expression differences, 11
Microarray platforms, overview of, and use in aquaculture, 6, 9
Microarrays
defined, 25
toxicogenomics and, 276–277
Microarray technology
applying in finfish aquaculture and research, 6, 7*t*–8*t,* 9–15
Atlantic cod 20K oligonucleotide microarray, 9
microarrray data analysis tools, 9–13
overview of microarray platforms, 6, 9
recommendations and future directions, 13–15
studying differential gene expression at high-throughput and, 25
Microencapsulation, islet transplantation and, 282
microRNA, 187
Microsatellite markers, shrimp breeding programs and, 22
Microsatellites
halibut QTL analysis and, 49
pedigree analysis and, 46
penaeid species and, 35
Mid-blastula transition, 177, *178*
in zebrafish, timing of, 180
Minimum Information About a Microarray Experiment, 13
Misgurnin, 106
distribution and spectrum of activity of, 109*t*
Misty somites, embryonic development in zebrafish and, 185
Mitochondrial transfer, clonal lines and ease of, 57–58
Molecular diagnostic test development, viral pathogens-Iridoviridae and, 86
Molt-inhibiting hormone, RNAi-based experiments in Penaeid shrimp and, 30*t*
Mononuclear phagocytes in fish, 208–214
primary cell culture methods for head kidney, 208–212
Moral reasoning, distinctive features in, 350
Moronecidin, 106
distribution and spectrum of activity of, 109*t*
Morpholino oligonucleotides, antisense, gene knock-down studies and, 181
Morpholinos, 88
functional analysis of SGIV genes with, 147
MOs. *See* Morpholino oligonucleotides
Mosaic expression pattern, in primary transgenic fish, 236
Mosaic hyperplasia, 294
Mosaicism
for fish and mammalian P1 transgenic organisms, 296–297
germline, F1 transgenic organisms and, 300
reducing, 314
Mozambique tilapia *(Oreochromis mossambicus)*
homologous promoters tested in, 247
hsp70 in, 242
MRE. *See* Metal response element
mRNA differential display, 22
Mud loach *(Misgurnus mizolepis),* transgene instability among populations of, 324
Multicolor transgenic biomonitoring fish, 275–276
Multtest, 10*t*
Muscle, in mammals, regulation of growth and differentiation of, 291
Muscle fibers, hyperplasia of, in transgenic fish, 298–299
Muscle growth/differentiation
in fish, myostatin and, 294
follistatin overexpression and, 296
localized, in P1 follistatin transgenic fish, 296–297
transforming growth factor-β superfamily members and, 291
Muscle mass, overexpression of follistatin and quadrupling of, in mice, 293

Muscle tissue, of GFP transgenic fish, GFP protein synthesis in, *297*
Mussel defensins and fragments, antiparasitic activity of, 110
Mutagens
  biomonitoring transgenic fish systems and, 270*t*
  models of biomonitoring fish and, 272–273
Mycobacterium marinum
  genomic sequencing of, 90*t*
  studying virulence genes of, 92
Myelopoiesis, in mammals, 208
Myostatin, 292, *292*
  deficiency and inhibition of, 291–293
    myostatin protein and, 292–293
    transforming growth factor-β muscle regulatory pathway and, 292
  fish and, 293–294
    muscle growth, 294
    TGF-β inhibition, 294
  inhibition of, and other TGF-β in mammals, 293
  secretion of, 293
Myostatin peptide, domains for, *293*
Myostatin protein, 292–293
Myticin, 106
  distribution and spectrum of activity of, 109*t*
Mytilin, 106
  antifungal activity of, 108
  distribution and spectrum of activity of, 109*t*
Mytimycin, 106
  distribution and spectrum of activity of, 109*t*
  fungal growth inhibited by, 108

N
Naked Cuticle, zebrafish homologs of, 183
nanos, RT-PCR analysis of, 201, *202*
National Center for Biotechnology Information, 186
Native habitats
  aquaculture and effects within, 327–328
    competition, 327
    interbreeding and introgression, 327–328
Natural killer cell enhancement factor, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Natural Sciences and Engineering Research Council, 283
Nature, noninstrumental *vs.* instrumental views of, 351
NCBI EST database, 22
Neo-DsRed fusion protein
  expression of, 200, *200*
  germ-line chimera production from zebrafish PGC cultures and, 203
"Neomales," 45
Neomycin, 166
Neurogenesis, maternal factors involved in, 185
New Brunswick hatchery, halibut broodstock pedigree analysis, 48*t*
Newfoundland winter flounder *(Pseudopleuronectes americanus)*
  antifreeze proteins and, *256*
  seasonal changes in AFP and AFP mRNA levels in, *257*
  tissue and cellular localization of AFP production in gill and skin epithelia of, *258*
Next-generation sequencing (NGS) technologies, 3, 13
Nile tilapia *(Oreochromis niloticus)*
  centromeric sequences identified in, 73
  conserved repetitive sequence found in telomeres of, 73
  number of ESTs in GenBank, 4*t*
  sex chromosomes and, 74
  transgene expression and transmission in, 236
Nimaviridae, 142
NimbleGen, 6
Nitrogenous compounds, marine aquaculture and accumulation of, 76
NMR spectroscopy. *See* Nuclear magnetic resonance (NMR) spectroscopy
Nocardia farcinica, genomic sequencing of, 90*t*
Nodal signals, Smad 2 and 3 and, 184
Nonnative habitats, genetic effects of farmed and transgenic animals in, 325–327
Normative components, ethical arguments tied to, 350
Normative premises, ethical arguments and, 350, 351, 352
Norwegian Biotechnology Advisory Board, 166
No tail gene, 196
Nova Scotia hatchery, halibut broodstock pedigree analysis, 48*t*
Novel viral proteins
  structural characterization of, 147, 149–150
    structural analysis of WSSV VP9, 149–150
    structural analysis of WSSV VP26 and VP28, 147, 149
    target selection and expression, 147
NR-13, *5*
NS, 157
NSERC. *See* Natural Sciences and Engineering Research Council
N-terminal signaling domain, for myostatin peptide, *293*
Nuclear magnetic resonance (NMR) spectroscopy, 84, 141
Nucleic acids, enhanced immune responses and, 129

O
Ocean pout *(Macrozoarces americanus)*
  AF(G)P in, 255
  antifreeze protein gene promoter in, 239
  contribution of antifreeze proteins to, *256*
Oligonucleotide microarrays, cDNA clones and, 94
OMPs. *See* Outer membrane proteins
1oxP sites, 244
OnMTGH1, growth hormone construct, localization on telomere of single acrocentric chromosome in transgenic coho strain 5750, *72*
Oocyte cryopreservation, 309–310
Oocyte (fish)
  maternal mRNAs in, 177
molecular portrait of, 178–181
    mRNA in oocyte and early embryo before mid-blastula transition, 179–181
    transcriptomic analysis of oocyte and early embryo, 178–179
mRNA in, before mid-blastula transition, 179–181
partially complete molecular picture of, 187
quality in, transcriptomic analysis of, 185–186
Oogenesis, 177
Open antimicrobial peptides, 106

Orange-spotted grouper iridovirus, 86
genus, accession number, and references, 85*t*
ORFO18R expression
functional analysis of SGIV genes with morpholinos and, 147
viruses and, 88–89
OSGIV. *See* Orange-spotted grouper iridovirus
Ostariophysi Siluriformes, examples of species with genomic resources and number of ESTs in GenBank, 4*t*
Outbred animals, crosses of clones to, 59
Outbreeding depression, 328
Outer membrane proteins, of *A. salmonicida,* further study of, 93
Ovaries, of triploid masu salmon recipients, into which rainbow trout spermatogonia had been transplanted, *230*
Oxidative stress, sperm cryopreservation and, 309

P
Pacific blue shrimp *(Litopenaeus stylirostris)*
AMP sequences identified in, 107
Pacific oyster *(Crassostrea gigas)*
AMP sequences identified in, 107
centromeric sequences identified in, 73
Pacific white shrimp *(Litopenaeus vannemei)*
AMP sequences identified in, 107
differential expression cloning studies in, 27*t,* 28*t*
genetic maps for, 35, 36
genomic research by EST analysis for, 22
microarrays with gene contents and, 25
panels of genetic markers developed for, 35
RNAi-based experiments in, 30*t,* 31*t,* 32*t,* 33*t*
PACs. *See* P1-derived artificial chromosomes
pAE6-Gvhsv, in vivo testing of, 160
PAHs. *See* Polycyclic aromatic hydrocarbons
PAMPs. *See* Pathogen-associated molecular patterns
Pancreases, donor, annual availability of, 281
Paracanthopterygii Gadiformes, examples of species with genomic resources and number of ESTs in GenBank, 4*t*
Pardaxin, distribution and spectrum of activity of, 109*t*
Parthenogenetic reproduction, of clonal fishes, 55
Pathogen-associated molecular patterns, 5, 122, 128, 208
cell culture methods for head kidney and, 211
trout macrophages studies and, 212, 214
Pathogens
identification of, in shellfish, fish, and wastewater generated by aquaculture, 76–77
resistance to, examples of experimental approaches to identify genes contributing to, *14*
Pathogen-specific microarrays, development of, 15
Pathway analysis programs, 12
Pattern formation, maternal factors involved in, 183–184
Pattern recognition receptors, 122, 128
PCA. *See* Principal component analysis
pCMV-IEP, 159
pCMV-Luc plasmid, 159
PCR amplicons, from transgenic fish, *295*
PCR-based assays, cDNA clones and, 94
PCR techniques, xii
pdnd-Neo-DsRed plasmid, diagram of, *200*
Pedigree analysis, for Atlantic halibut, 46–47, 48*t*
Penaeidins, 106
distribution and spectrum of activity of, 109*t*
Penaeid shrimp
advances in genomics and genetics of, 21–37
differential expression cloning studies in, 26*t*–28*t*
DNA vaccine for WSSV in, 158
RNAi-based experiments in, 30*t*–33*t*
studies reporting characterization of ESTs in, 23*t*–24*t*
value of, for worldwide aquaculture, 21
Penaeus monodon
differential expression cloning studies in, 26*t,* 27*t,* 28*t*
feeding or bacteria-mediated RNAi and, 34
genetic maps for, 35
genomic research by EST analysis for, 22
ICAT identification of differentially expressed proteins in WSSV-infected subcuticular epithelium of, 146
microarrays with gene contents and, 25
panels of genetic markers developed for, 35
RNAi-based experiments in, 30*t,* 31*t,* 32*t,* 33*t*
Penicillium crustosum, AMPs and inhibition of, 108
Perciformes, selected microarray platforms, overview of, 7*t*
Perkinsus marinus, tachyplesin I active against, 110
Peroxisome proliferator response element, response of, to environmental pollutants, 276*t*
Persistent organic pollutants
biomonitoring transgenic fish systems and, 270*t*
models of biomonitoring fish and, 272
PERV. *See* Porcine endogenous retrovirus
Pesticides, aquaculture and increased use of, 349
PGCs. *See* Primordial germ cells
Phenotypic expression, 324–325
Photobacterium damselai subsp. *piscida,* vaccines for, 153
Phylogenetic footprinting, 239
Piedmontese myostatin gene, 291
Pili, 92
Pink salmon, sex chromosomes and, 75
Piscidin, 106
Candida albicans inhibited with, 108
distribution and spectrum of activity of, 109*t*
Piscidin 2, antiparasitic activity of, 110
Piscine muscle growth, TGF-β signaling and regulation of, 300
Piwi-interacting RNAs, 187
Plasmid DNA
formulating, 161–162

Plasmid DNA (*Continued*)
long-term persistence of, in injected fish/shellfish, 159
persistence and distribution of, 166
Plasmid profiling, *A. salmonicida* studies and, 91
Platelet-derived growth factor, 88
Pleurocidin, 112, 113
Candida albicans inhibited with, 108
cytotoxic activity of, 110
distribution and spectrum of activity of, 109*t*
fish-derived, 106
Pleuronectiformes, selected microarray platforms, overview of, 7*t*
pMCV1.4-Gvhsv, in vivo testing of, 160
Poikilothermic animals, heat shock-induced gene expression in, 247
Polar/subpolar oceans, temperature of fish residing in, 253
Pollutant classes, mutational spectra and, 272
Pollutants. *See also* Environmental pollutants
aquaculture and increase in, 349
Polycyclic aromatic hydrocarbons, 272
Poly-*L*-lysine, cell culture methods for satellite cells and, 215
Polymerase coding gene, RNAi-based experiments in Penaeid shrimp and, 32*t*
Polyphemusin, 106
distribution and spectrum of activity of, 109*t*
fungal growth inhibited by, 108
Polysaccharides, enhanced immune responses and, 129
P1-derived artificial chromosomes, 241
P1 follistatin transgenic fish
juvenile, inner abdominal wall of, *299*
localized muscle growth in, 296–297
P1 transgenic fish, increased condition factor in, 297
POPs. *See* Persistent organic pollutants
Porcine endogenous retrovirus, 283, 284
"Position effect," transgenesis efficiency and, 238
Position/integration effects, phenotypic expression and, 324
Pou5fl/Oct4, pluripotency of embryonic stem cells and, 185
PPRE. *See* Peroxisome proliferator response element
Predation, ecological effects of, 327
"Pre-genotyped" inbred lines, 56
Primary cell cultures
advantages with, 207–208
shrimp and in vitro delivery of, 34
Primary transgenic animals, established transgenic lines and, 236
Primordial germ cells, 180, 181
cryopreservation of, 312–313
isolating, difficulty with, 227
of rainbow trout, transfected with vasa-GFP gene, 224, *225*
specification of, 196, 198
Principal component analysis, 12
Probiotics, 110
Prodomain, for myostatin peptide, *293*
Production waste, aquaculture and increase in, 349
Prohibitins, role of, 186
Prohibitin 2, unfertilized rainbow trout eggs and, 185
Prolactin, DNA vaccine vector construction and, 159
Proline-rich AMPs, 106
Promoter region
in genes, *240*
in inducible gene expression systems, 244
transgene expression and, 239
transgenic fish production and, 241
Prophenoloxidases, RNAi-based experiments in Penaeid shrimp and, 31*t*
Protacanthopterygii Salmoniformes, examples of species with genomic resources and number of ESTs in GenBank, 4*t*
Protamine, DNA vaccine vector construction and, 159
Protease coding gene, RNAi-based experiments in Penaeid shrimp and, 32*t*
Protein engineering, for enhancing AFP activity, 262, *262*
Protein function, structural analysis of, 141
Protein kinase coding gene, RNAi-based experiments in Penaeid shrimp and, 31*t*
Protein quantification, proteomic studies and, 143
Protein(s)
human nutrition, marine animals and, 348
transgene-encoded, 235
2D PAGE and separation of, 84
Protein separation technologies, 141
Proteome, defined, 84
Proteomics
A. salmonicida bacterial pathogens and, 92–93
description of, 142–143
fish health studies and application of, 84
host, fish health and, 96
vaccine development and, 97–98
viral pathogens-Iridoviridae and, 87–89
ProVale, 130
PRRs. *See* Pattern recognition receptors
Pseudomonas fluorescens, transgenic expression and resistance against, 112
pTG-Neo-DsRed-ntl, targeted incorporation of, into zebrafish ES cells, *197*
pTG-Neo-DT-ntl, targeted incorporation of, into zebrafish ES cells, *197*
pTG-pac-DsRed-DT-hag, diagram of, *198*
PubMed, 6
Puromycin (pac) selection, DsRed and, *198*
Putative farnesoic acid O-methyltransferase, RNAi-based experiments in Penaeid shrimp and, 30*t*
Putative ion transport peptide, RNAi-based experiments in Penaeid shrimp and, 30*t*

Q
QPCR. *See* Quantitative reverse transcription-polymerase chain reaction
qPCR
assessing adaptive immune response, 129
quantitative PCR integrating pathogen virulence and immune response, 129
suppression subtractive hybridization and, 129

QPCR assays. *See* Quantitative reverse transcription-polymerase chain reaction assays
Quality control, of array data, 10
Quantitative revere transcription-polymerase chain reaction assays, *5*
Quantitative reverse transcription-polymerase chain reaction, 13, 14, 178
Quantitative trait loci
  clonal lines and, 58
  domestication, clonal line approach and, 64
  for halibut, 49
Quantitative trait loci studies, genetic mapping and, 60
Quantitative trait locus (QTL) mapping, 47
  halibut and
    sensitivity of, 49–50
    status of, 50

R
Rab-GTPase, RNAi-based experiments in Penaeid shrimp and, 31*t*
RACE. *See* Rapid amplification of cDNA ends
RAGs. *See* Recombination-activating genes
Rainbow trout chromosome Omy7, localization of ID1B gene to, *72*
Rainbow trout eggs, production statistics for those injected with follistatin DNA construct, 296*t*
Rainbow trout follistatin coding sequence, schematic of *mylc* expression vector, *295*
Rainbow trout karyotype, localization of 5S rRNA genes in, *72*
Rainbow trout mononuclear phagocytes, microscopic changes in, undergoing spontaneous differentiation under in vitro conditions, *210*
Rainbow trout *(Oncorhynchus mykiss),* 3
  accelerated production of clonal lines in, 57
  BAC libraries for, 75
  blastomere cryopreservation and, 312
  chromosome identification in, 73
  clonal, genetic mapping and, 59–60
  clonal lines of, 55
  cytokines as adjuvants for DNA vaccines and, 161
  dissection and isolation of head kidney cells from, *209*
  domesticated, clonal lines from, 64
  effects of follistatin overexpression in, 300
  embryos (alevins) obtained from male masu salmon parents after transplanting primordial germ cells from, *226*
  embryos obtained from female masu salmon parents, *228*
  follistatin construct and, 294–295
  freezing point of (without AF(G)P), 255
  gene expression-based toxicogenomics approach applied to, 277
  germ cell transplantation and, 223, 224
  growth of wild and farmed, to transgene OnMTGH1, 324
  immune system in, 121
  length of time plasmid DNA detected in, 159
  liposome formulations and, 163
  localization of rDNA on long arms of chromosome 20 in, 71
  microarray platforms for, 7*t*
  microinjection and transgenic screening of, 295–296
  as model organism, TGF-β superfamily and, 294
  muscle satellite cells, differentiation of, cultured at 18° C with DMEM containing 10% FBS, *215*
  muscle satellite cells from, 216
  number of ESTs in GenBank, 4*t*
  primordial germ cells of, transfected with vasa-GFP gene, 224, *225*
  sea water-adapted, efficacy of type 1 and spruce budworm AFP at improving freeze resistance of, *261*
  spermatogonia from, transplanted into masu salmon, 227–228
  sperm extenders used for, 308
  transcriptomic analysis of oocyte quality in, 185, 186
  transcriptomic analysis of unfertilized eggs of, 179
  transgene expression and transmission in, 236
  transgenic, overexpression of follistatin in, 291
  transplanted spermatogonia from and embryos obtained by fertilization of egg and sperm from triploid masu salmon, *230*
  viral DNA vaccines for, 155*t,* 156*t*
  viral hemorrhagic septicemia in, 156
Rainbow trout spleen, blastula-stage zebrafish embryo, RTS34st cell line derived from, *194*
Ranavirus, 142
  full genome sequences available for, 85
Randomly amplified polymorphic DNAs (RAPDs), penaeid species and, 35
Rapid amplification of cDNA ends, bidirectional, *5*
RAREs. *See* Retinoic acid response elements
RBIV. *See* Rock bream iridovirus
Recombinant DNA, xii
Recombinant inbred lines, 56
Recombinant vaccines, xi
Recombination, Cre-mediated site-specific, 244
Recombination-activating genes, 123
Recombination between sexes, halibut linkage map and, 49
"Redband" varieties, high temperature tolerance among, 63
Red fluorescent protein, 196, *198,* 275
Red Queen hypothesis, 132
Red seabream iridovirus, 86
  genus, accession number, and references, 85*t*
  viral DNA vaccines against, 155*t*
Red seabream *(Pagrus major)*
  ES-like cell lines established for, 195
  viral DNA vaccines for, 155*t*
Rejection of transplanted cells, overcoming, 224–225
Relative percent survival, DNA vaccines and, 157, 158
Renibacterium salmoninarum ATCC 33209, genomic sequencing of, 90*t*
Reporter genes, 235
Repressor binding sites, 239
Reproduction control, 347
Reproductive technologies
  cryobanking combined with, 313

Reproductive technologies (*Continued*)
oocyte cryopreservation and, 309–310
sperm cryopreservation and, 307–309
Response elements (REs), 275
response of, to environmental pollutants, summary, 276*t*
Restriction endonuclease fingerprinting, *A. salmonicida* studies and, 91
Restriction enzymes, xii
Restriction fragment length polymorphisms
pedigree analysis and, 46
penaeid species and, 35
Retinoic acid response elements, response of, to environmental pollutants, 276*t*
Retinoid X response elements, response of, to environmental pollutants, 276*t*
Return on investment, gene biotechnologies for commercial aquaculture and, xii
Reverse transcriptase, xii
RFLPs. *See* Restriction fragment length polymorphisms
RFP. *See* Red fluorescent protein
Ribosomal DNA (rDNA)
characterizing karyotypes of scallops and fish with probes to, 73
localization of, in particular aquaculture strains, 71–72
repetitive sequences and, 71
RILs. *See* Recombinant inbred lines
RNA, clones and utilization of, 60–61
RNAi-based applications
methods to trigger gene silencing by, in shrimp, 29, 34–35
feeding or bacteria-mediated RNAi, 34
injection of dsRNA, 29, 34
in vitro delivery to primary cell cultures, 34
in shrimp aquaculture, 29, 34–35
RNAi-based experiments, in Penaeid shrimp, 30*t*–33*t*
RNA interference (RNAi), 21
gene function and, 89
gene knockdowns and, 288
as tool to promote antiviral protection in shrimp, 34–35
as tool to unravel gene function in vivo, 34
Roche Genome Sequencer FLX system, 35
Rock bream iridovirus, 86
genus, accession number, and references, 85*t*
rpl64 gene, early embryonic development in zebrafish and, 186
RPS. *See* Relative percent survival
RSIV. *See* Red seabream iridovirus
RTS34st feeder cells, pluripotency of zebrafish ES cells maintained by, 195
RXRES. *See* Retinoid X response elements

S
SAGE. *See* Serial analysis of gene expression
Salinity tolerance, 347
Salmon
freeze-resistant, future directions in production of, 260–263
increasing functional levels of AFP, 260–263
tissue-specific expression, 263
Salmonella, live attenuated bacteria and, 128
Salmonid aquaculture, temperature tolerance and, 62–63
Salmonid clonal lines, utility of, 55
Salmonid fish farms, females preferred by, 74
Salmonid growth enhancement case study, 328, 334–335
comparing growth-selected and GH-enhanced phenotypes, 328, 334–335
Salmonid populations
fitness-related trait divergence between wild and farmed, GH transgenic or GH-treated salmonids compared in natural or near-natural lab environments, 331*t*–333*t*
fitness-related trait divergence between wild and growth selected, GH transgenic or growth hormone-treated *vs.* artificial tanks or aquaria, 329*t*–330*t*
Salmonids
cell lines from ATCC available for, 207
families of centromeric sequences in, 73
humoral immune response in, 121
indirect feminization approach and, 45
microarray platforms available for, 6
sperm cryopreservation studies for, 307
Salmonid species
aquaculture diets fed to, 61
generation times for, 56–57
Salmoniformes, selected microarray platforms, overview of, 7*t*
Salvelinus genus, families of centromeric sequences in species of, 73
SAM. *See* Significance Analysis of Microarrays
Sampling, limited, clonal lines and, 58
Sandoz Canada, 283
Sandoz USA, 283
Satellite cells
primary cell culture methods for, 214–216
trout muscle, primary cell culture methods for, 214–216
SAVR. *See* Systemic antiviral responses
Scallops, types of karyotypes in, 73
SCF. *See* Stem cell factor
Scientific research, interests of aquaculture industry aligned with, 347
Scottish hatchery, halibut broodstock pedigree analysis, 48*t*
Sdf1a, recombinant, growth of zebrafish PGCs and, 199, *199*
Sdf1b, recombinant, growth of zebrafish PGCs and, 199, *199*
SDF1/Sdf1, PGCs and, 199
SD locus, salmonid fish species and, 74–75
SDS-PAGE, 84
Seabass *(Dicentrarchus labrax),* sperm extenders used for, 308
Sea cage aquaculture, low temperature limitations to, 258–259
Seafood products, capacity of fishing industry and annual demand for, 348
Sea perch, ES-like cell lines established for, 195
Sea squirt *(Ciona intestinalis),* AMP ESTs in hemocyte libraries identified in, 107
Self-organizing maps, 12
Senegalese sole *(Solea senengalensis)*
microarray platforms for, 7*t*
oligo-based microarrays for, 94

Sequence data, functional information from, 86–87
Sequence-specific gene silencing, gene function and, 89
Serial analysis of gene expression, 178
17alpha-methyldihydrotestosterone, sex control in teleosts and, 45
17alpha-methyltestosterone, 45
17β-estradiol, sex control in teleosts and, 45
Sex chromosomes, identification and characterization of, 74–75
Sex determination, for Atlantic halibut, 45
Sex reversal, in Atlantic halibut, 45–46
Sexual dimorphism, clonal fish and, 64–65
SGIV. *See* Singapore grouper iridovirus
SGIV genes, morpholinos and functional analysis of, 147
SGIV proteins, structural and nonstructural, *144*
Shellfish. *See also* DNA vaccines, for viral diseases of farmed fish and shellfish
  cultured, pathogens identified in, 76–77
Short interfering RNA (siRNA), 89, 187
  injections of, in shrimp, 29
  RNAi-based experiments in Penaied shrimp and, 33*t*
Shotgun proteomics, 142, 143–144
Shrimp
  analytical challenges in genomics and genetics of, 36–37
  EST collection as approach to gene discovery in, 21–22
  genetic maps and, 35–36
  large insert size genomic libraries and, 36
  medium- to high-throughput studies of differential expression and gene discovery in, 22, 25, 29
  penaeidins isolated from, 106
  single nucleotide polymorphisms and, 35
  viral DNA vaccines for, 156*t*
  white spot syndrome virus in, 141–142
Shrimp aquaculture
  feeding or bacteria-mediated RNAi and, 34
  methods to trigger silencing in shrimp, 29, 34–35
    injection of dsRNA, 29, 34
    in vitro delivery to primary cell cultures, 34
  RNAi-based applications in, 29, 34–35
Shrimp epithelia, WSSV-infected cells in, 142
Shrimp farming, infections and, 34–35
Siggenes, 10*t,* 11
Significance Analysis of Microarrays, 9, 10*t,* 11
Silencers, 239
Siluriformes, selected microarray platforms, overview of, 8*t*
Singapore grouper iridovirus, 141
  description of, 142
  functional information from sequence data and, 86
  genus, accession number, and references, 85*t*
  viral proteomics of, 143
Single nucleotide polymorphisms, *14*
  genotyping platforms, 3
  pedigree analysis and, 46
  shrimp and, 22, 35
"Six pack" phenotype, 297, 300
  in P1 juvenile transgenic fish overexpressing follistatin, 296, *298*
  2-year-old P1 transgenic fish with, *298*
Skin-type AFP, liver-type AFP *vs.,* 257
Skin type AFP mRNA expression, distribution of, on gill filament lamellae, *258*
Sleepy grouper disease, 142
Smad2/3 activities, 184
Snake-head rhabdovirus, antiviral activity of AMPs and, 108
SNPs. *See* Single nucleotide polymorphisms
Social development, aquaculture industry and, 348
Sockeye salmon *(Onchorhynchus nerka)*
  number of ESTs in GenBank, 4*t*
  sex chromosomes and, 75
  viral DNA vaccines for, 155*t*
sodA, 92
sodB, 92
SOLiD, 35
SOLiD 3, 13
Somite boundary foundation, in zebrafish, 185
Somitogenesis, maternal factors involved in, 185
SOMs. *See* Self-organizing maps
Sonic Hedgehog gene *(shh),* 184, 239
Spawning channels
  for chinook salmon, *131*
  hatcheries and use of, 131
Spawning records, errors in, pedigree analysis and, 47
Species invasions, ecology, biodiversity and, *326*
Sperm
  cryopreserved, clonal line approach and, 57
  freezing and thawing protocols, steps in, 307–308
Spermatogonia
  sexual plasticity of, 227
  transdifferentiation of, from adult fish into eggs, 227–228
Sperm banks, for conservation purposes, 314
Sperm collection, 307
Sperm cryobanks, 307, 313
Sperm cryopreservation, 203–204, 307–309
Sperm freezing, 308, *309*
Sperm loading, 308
Sperm quality analysis, 307
Sperm thawing, *309*
Spring ice conditions, maximum, in Atlantic Canada, *259*
Spring viremia of carp virus, viral DNA vaccines against, 155*t*
Spruce budworm AFP, improving freeze resistance of rainbow trout and, *261*
Squint, recent work on, 184
SSH. *See* Suppression subtractive hybridization
SSH cDNA libraries. *See* Suppression subtractive hybridization cDNA libraries
Statistical analysis of gene expression differences, 11
Stellata sturgeon *(Acipenser stellatus),* dispermic androgenesis in, 314
Stem cell factor, culture of pluripotent mammalian EG cells and, 195
Stem cells, self-renewal of, 193
Stem cell technology, application of, to marine biotechnology, 203–204
Stickleback
  gene expression-based toxicogenomics approach applied to, 277
  genome sequences available for, xii
Streptococcus iniae, genomic sequencing of, 90*t*

Sturgeon
germ cell transplantation and, 223
sperm cryopreservation studies for, 307
Styelin, 106
distribution and spectrum of activity of, 109*t*
Subunit vaccines, xi
Sucrose, embryo cryopreservation and, 311
Support vector machines, 36, 37
Suppression subtractive hybridization, 22, 25
antimicrobial peptides and, 107
qPCR and, 129
Suppression subtractive hybridization cDNA libraries, 4, *5,* 13
Surrogate parents, germ cell transplantation and, *224*
Surrogate parent technology, prospective uses for, 229–230
Survival rates, clonal lines and, 58
Sustainable development principle, 351
SVCV. *See* Spring viremia of carp virus
SVMs. *See* Support vector machines
Swanson clonal line, of rainbow trout, 57
Swanson doubled haploid clonal line, in rainbow trout genome project, 61
SWI/SNF chromatin remodeling complex, 185
Systemic antiviral responses, 165

T
Tachycitin, distribution and spectrum of activity of, 109*t*
Tachyplesin, 106, 108, 110
distribution and spectrum of activity of, 109*t*
fungal growth inhibited by, 108
Tachyplesin 1, antiparasitic activity of, 110
Tap pili (tapA), bioinformatic analysis of *A. salmonicida* and, 92
Taxonomy Browser (NCBI), 13
TCDD, 272
TCDD-targeted tissues, identifying, transgenic zebrafish embryos and, 274
T-cell antigen receptors (TCRs), 123
T-cytotoxic lympohcytes, 160
Teleosts
diversity among, 121
improving transgenesis in, 247
sex control in, 45
species with genomic resources and number of ESTs in GenBank, 4*t*
transposable elements in, 73
Teleost species, commercial rearing of monosex stocks of, 44
Temperature tolerance, 62–63, 347. *See also* Antifreeze proteins; Freeze resistance; Heat stress resistance
Temporal control of transgene, heat shock system and, 247
Tenebrio AFP, ice-binding motif with, 262
Testis triploid masu salmon, cross sections of, *229*
Tetracycline-off system, for induction of transgene expression, *245*
Tetracycline regulation system, inducible systems and, 244
Tetracyline-dependent tTA, 244, *245*
Tetraodon, genome sequences available for, xii
Tetraodon nigroviridis, transposable elements localized in, 73
tet regulatory elements, 244
TFs. *See* Transcription factors
TGF-β inhibition in fish, myostatin and, 294
TGF-β ligands, activin receptor signaling pathway utilized by, *292*
TGF-β signaling, regulation of piscine muscle growth and, 300
Tg(mvtg1:gfp) transgenic medaka, *271*
Thawing, of sperm, 308
T-helper cells, 160
Thermal hysteresis, 255, *262*
Thermal tolerance, clonal lines and, 62–63
34 dDa AFP, winter flounder and secretion of, 256
Thyroid hormone response element, response of, to environmental pollutants, 276*t*
Tilapia *(Oreochromis aureus)*
ActRIIB protein injection and increased size of, 294
clonal lines of, 55
dechorionating embryos of, 287
Titin-cap, 293
TNF. *See* Tumor necrosis factor
TNF-alpha
EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
microscopic changes in rainbow trout mononuclear phagocytes and, *210*
studies using trout macrophages and, 212
TNF decoy receptor, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
To12-mediated transgenesis, generating transgenic zebrafish with use of, 236, *237,* 238
To12 transposable element, 236
Tourism, aquaculture and, 349
Toxicogenomics, microarrays and, 276–277
Toxicological screening, fluorescent transgenic embryos/larvae used for, *273,* 273–274
Toxicology, aquatic, small fish models as sentinels in, 268
Trait-relevant gene discovery, targeted, in finfish, 4–6
Transcription factors, 239
Transferrin, DNA vaccine vector construction and, 159
Transforming growth factor beta superfamily, of secreted cytokines, embryogenesis signaling and, 183–184
Transforming growth factor-β muscle regulatory pathway, description of, 292
Transforming growth factor-β superfamily members, muscle growth/differentiation and, 291
Transgene expression in fish
Cre recombinase and activation of, *246*
regulation of, 239, 347
spatial and temporal regulation of, 235–247
tetracycline-off system for induction of, *245*
Transgene insertion site, location of, 238
Transgene integrants, detection of, xii
Transgenes
defined, 235
FISH and, 71–74
hypermethylation of, 238
phenotypic expression and influence of, 335

Transgenesis, phenotypic enhancement strategy of, *vs.* intentional selection, 323
Transgenesis efficiency, limitations in, 238–239
Transgene X, *243, 245, 246*
Transgenic animals, lower element of predictability in, *vs.* in farmed animals relative to risk assessment, 335
Transgenic biomonitoring fish
  advantages of, 274–275
  early, development of, 268
  multicolor, 275–276
Transgenic expression, AMPs, disease-resistant species for aquaculture and, 112–113
Transgenic fish, 235–236
  agarose gel showing PCR amplicons from, *295*
  creating, most common method of, 323
  cryopreservation and, 314
  designer promoters in, 275
  environmental monitoring and toxicology applications with, 267–277
  hyperplasia of muscle fibers in, 298–299
  muscle sections (representative) from, *300*
Transgenic fish project, history of, funding and patent issues, 282–284
Transgenic lines, established, primary transgenic animals and, 236
Transgenic organisms, phenotypic expression of, 324–325
Transgenic tilapia
  chimeric hatchling developing from noninjected zygote, *288*
  future improvements to, 287–288
  negative control hatchling developing from noninjected zygote, *288*
  positive control hatchling developing from TRITC-injected zygote, *288*
  production of, 284–286
  status of breeding program for, 286–287
  for xenotransplantation, 281–289
Transgenic trout
  follistatin overexpression, transgenic F1 offspring and future of, 300
  follistatin overexpression and production of, 294–296
Transglutaminase, RNAi-based experiments in Penaeid shrimp and, 31*t*
Transposable elements, localization of, in model fish genomes, 73
Transposase-mediated transgenesis, 236–238
Transposon-mediated gene transfer, 247
Transposons
  defined, 236
  FISH and, 71–74
TreeView, 12
TREs. *See* Thyroid hormone response elements
Trihodina sp., piscidin 2 active against, 110
Triploid fish
  male and female, sterility of, 228
  sterile, but with normal somatic cells, 228–229
  sterility of, reason for, 229
Triploid masu salmon recipients, ovaries of, into which rainbow trout spermatogonia had been transplanted, *230*
TRITC-labeled grey tilapia blastula cells, larvae examined for presence and distribution of labeled cells, 287, *288*
Trout, antifreeze proteins and, *256*
Trout macrophages, results of studies using, and impact on aquaculture, 212, 214
Trout mononuclear phagocytes, primary, phagocytosis of zymosan by, *211*
Trout muscle satellite cells, 214–219
  primary cell culture methods for, 214–216
  subcellular localization of endogenous GLUT4 in, *218*
  time course of insulin-stimulated 2-deoxyglucose uptake in, *217*
Trout Mx1 promoter, DNA vaccine vector construction and, 160
Trout protein 1, differentially regulated, EST size, accession # of similar protein, species most similar to, BLASTx E-value, and clones in 12 hour and 30 hour ESTs, 213*t*
Trypanosoma brucei, mussel defensins and fragments active against, 110
T7-Pac-DsRed-nanos-3′ UTR construct
  cDNA encoding with, 201
  diagram of, *201*
Tumor necrosis factor, 88, 160
Turbot embryo, at tail bud, showing different envelopes and compartments, *310*
Turbot *(Psetta maxima),* microarray platforms for, 7*t*
Turbot *(Scophthalmus maximus)*
  oligo-based microarrays for, 94
  primary cell culture methods for head kidney from, 212
  sperm cryopreservation studies for, 307
  sperm extenders used for, 308
  viral DNA vaccines for, 156*t*
Two-dimensional gel electrophoresis, 142
Two-dimensional polyacrylamide gel electrophoresis (2D PAGE), 84
Type II ethical problems, defined, 346
Type III secretion system, *A. salmonicida* and, 91, 94

U
UAS. *See* Upstream activating sequence
Unigene human gene symbols, 12
Unintentional selection, 325
Unisex populations, industry preference for, 74
Unsupervised hierarchical clustering, 11
Upstream activating sequence, 242
Utilitarian principle, 351

V
V. anguillarum, transgenic expression and resistance against, 112
Vaccine adjuvants, 112
Vaccine development
  genomics, proteomics and, 97–98
Vaccines, xi. *See also* DNA vaccines, for viral diseases of farmed fish and shellfish
  attenuated, 98
  knowledge about fish immunity and, 127–129
    adjuvants, 128
    live attenuated bacteria as carriers, 128–129
    method of delivery, 128

vasa gene
germ cell expression of, 223–224
RT-PCR analysis of, 201, 202, *202*
vasa-GFP genes
expression of, in spermatogonia of male adult fish, 227
in newly hatched embryos, 226
vasa RNA, germline establishment and, 181, 183
Vascular endothelial growth factor, 88
Vector construction, DNA vaccine formulations and, 158–160
VEFG. *See* Vascular endothelial growth factor
VHSV. *See* Viral hemorrhagic septicemia virus
Vibrio alginolyticus, genomic sequencing of, 90*t*
Vibrio anguillarum, 128
AMPs and protection against, 111
Vibrio campbellii, genomic sequencing of, 90*t*
Vibrio harveyi, genomic sequencing of, 90*t*
Vibrio ordalii, vaccines for, 153
Vibrio parahaemolyticus, genomic sequencing of, 90*t*
Vibrio splendidus, genomic sequencing of, 90*t*
Vibrio vulnificus, genomic sequencing of, 90*t*
Viral diseases
aquaculture and, 141
shrimp farming and control of, 34–35
Viral envelope proteins, infection and, 147
Viral hemorrhagic septicemia virus, 154
antiviral activity of AMPs and, 108
injection-site EAVR and, 164
late anitiviral responses and, 165
in rainbow trout, 156
systemic antiviral responses and, 165
vaccine efficacy trials reported for, 156
viral DNA vaccines against, 155*t,* 156*t*
Viral pathogens
Iridoviridae, 85–89
functional information from sequence data, 86–87
genomic studies, 85–86
molecular diagnostic test development, 86
proteomics, 87–89
Viral promoters, 242
Viral replication, LITAF and, 88
Viral vaccines, basic formulations used for, 153
Virtue ethics, 352
Vitellogenin receptor, RNAi-based experiments in Penaeid shrimp and, 30*t*
Vitellogenins, xenoestrogens and, 269
Vitrification, 306, 311, 312
VivoRx, 283
VP2, 157
VP3, 157
VP9, structural analysis of, 149–150
Vp15, RNAi-based experiments in Penaeid shrimp and, 32*t*
Vp19, RNAi-based experiments in Penaeid shrimp and, 32*t*
VP16 trans-activator, 244
VP26
localization and structure of, *148*
structural analysis of, 147, 149
VP28, 158, 159
localization and structure of, *148*
RNAi-based experiments in Penaeid shrimp and, 31*t,* 32*t*
structural analysis of, 147, 149
VP281, RNAi-based experiments in Penaeid shrimp and, 31*t*

W
Walk-back method, aquaculture and, 50
Water pollution detection, transgenic biomonitoring fish and, 274
Wetlands, aquaculture and impact on, 349
White spot syndrome virus, 108
clinical signs of, 142
description of, 141–142
DNA vaccines and, 154, 156*t,* 158
ICAT identification of differentially expressed shrimp proteins and, 145
localization of structural proteins in, 146*t*
viral proteomics of, 143–145
characterization of viral protein components, 143–144
iTRAQ for viral protein localization in, 145
Whole chromosome painting, 75
Whole genome sequencing, clonal lines and, 61
Whole inactivated (killed) vaccines, 153
Wild populations, interbreeding of farmed populations and, 327
Wingless signaling, developmental processes controlled by, 183
Winter flounder, 263
AF(G)P in, 255
discovery of hyperactive AFP from, 263
increasing functional level of AFP and, 260–261
as model for antifreeze protein freeze resistance strategies, 255–258
vitrification study and, 311–312
WSSV. *See* White spot syndrome virus
WSSV proteins, structural and nonstructural, *144*

X
Xenobiotic compounds, biomonitoring transgenic fish systems and, 270*t*
Xenoestrogens, 269
Xenotransplantation, 347
clinical islet, producing ideal donor tissue for, 288
PGC-mediated gene manipulation combined with, 198
regulation of, 283
transgenic tilapia for, 281–289
Xenozootic diseases, islet transplantation and, 282
X-ray crystallography, 141

Y
Yellow fluorescent protein, 275
Yellow perch *(Perca flavescens)*
number of ESTs in GenBank, 4*t*
primary cell culture methods for head kidney from, 212
Yellowtail flounder *(Limanda ferruginea),* antifreeze proteins and, *256*
Yersinia ruckeri
genomic sequencing of, 90*t*
vaccines for, 153
YHV-binding protein, RNAi-based experiments in Penaeid shrimp and, 31*t*
YHV-protease, RNAi-based experiments in Penaeid shrimp and, 32*t*
Yolk-sac resorption (YSR), developmental success assessed through embryonic survival at eyed stage and percentage of normal alevins at, *186*

Z
Zebrafish *(Danio rerio),* 89
beta-catenin expression and, 183
Brg1 in, 185
chemokines in, 127
chilling sensitivity in, 311
clonal lines of, 55
Cre recombinase system tested in, 245
cytokines as adjuvants for DNA vaccines and, 161
derivation of cell cultures from, 194–196
enhancer and gene-trapping strategies and, 242
establishment of, as vertebrate model, xii
Gal4 enhancer trap lines active in neural circuits of, 243
GDF-11 ortholog discovered in, 294
gene expression-based toxicogenomics approach applied to, 277
genome sequences available for, xii
heat shock response tested in, 247
Irf6 and differentiation of enveloping layer in, 184
length of time plasmid DNA detected in, 159
MBT in, 180
microarray platforms for, 6, 7*t*
misty somites and embryonic development in, 185
most complete microarray platforms for, 13
multicolor transgenic, 276
oligo arrays, reducing redundancy of platforms for, 13
oocyte cryopreservation studies and, 310
processed microRNAs in, 180
role of maternal mRNAs in oocyte quality and early embryonic development, 182*t*
as sentinels in aquatic toxicology, 268
To12-mediated transgenesis and generation of, 236, *237,* 238
transcriptomic analysis of oocyte and early embryo in, 178–179
transcriptomic analysis of oocyte quality in, 186
transgene expression and transmission in, 236
transgenic, GATA-2 gene and, 241
vasa RNA expression in, 183
vertebrate development investigations and, 179
vtg mRNAs inducible in, 269
Zebrafish embryonic development, maternal and zygotic gene activities during, *178*
Zebrafish ES cells, targeted incorporation of pTG-Neo-DsRed-ntl or pTG-Neo-DT-ntl into, *197*
Zebrafish PGC cultures, 198–199
derivation of, 196, 198–202
germ-line chimera production from, 202–203
Zebrafish Sdf1, growth of zebrafish PGCs and, 199
Zhikong scallops, type of karyotype in, 73
Zinc-finger nucleases, targeted deletions introduced into zebrafish genome with, 203
ZtA6 cells, spermatogonial stem cells in zebrafish and, 195–196
Zygotic transcription, activation of, 177
Zymosan, phagocytosis of, *211*