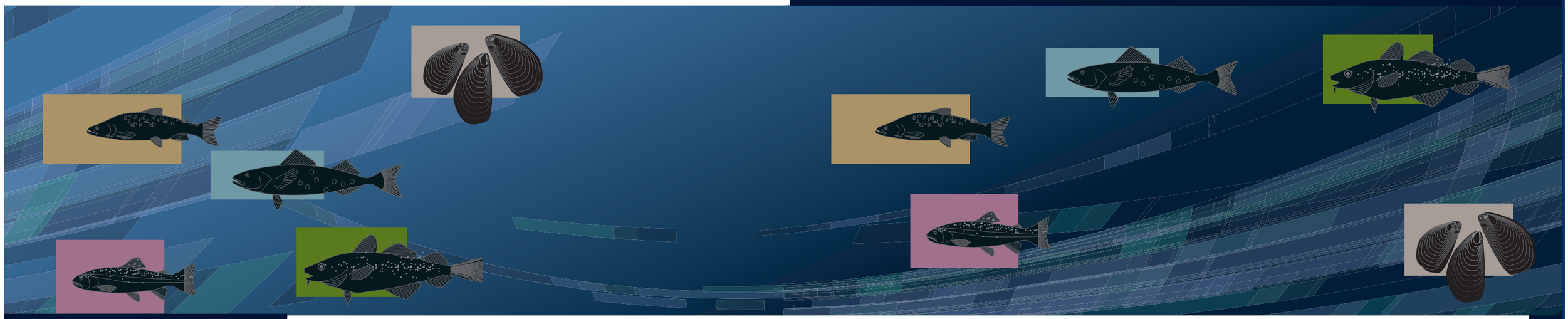
107-3 2009

# Application of Genome Science to Sustainable Aquaculture

**Bulletin** of the Aquaculture Association of Canada



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# Fostering a Healthy Canadian Aquaculture Industry

Canada is poised to be a leader in the growing and important global aquaculture industry.

We all know that a major component of a successful aquaculture practice is a healthy and strong broodstock. Improved breeding populations can help fish farmers avoid the time- and money-consuming challenges of disease, environmental stressors and extensive grow out times. This can make or break the ability to compete in an industry that is expected to have a demand of 40-million metric tonnes by 2030.

Genomics research is already having a positive impact on Canadian aquaculture viability. Much of that has come from Atlantic Canada.

Through Genome Atlantic projects in halibut and cod, the aquaculture industry in Atlantic Canada has reaped the benefits of greatly enhanced broodstock programs as researchers have identified specific genetic markers related to valuable traits such as growth rate and disease resistance.

Now, our industry partners have the genomic information to make informed selections for breeding, and can save precious time and resources.

We are about to begin a new project that will look at the industry challenge of early sexual maturation in cod — a challenge that can cause a loss of $1.6 million in a 500,000-fish cage site. Our project will attempt to alleviate this problem by creating sterile offspring from the elite broodstock of our earlier cod project.

At Genome Atlantic, we are proud of the genomics research that is helping the aquaculture industry meet consumer demand for a healthy, tasty and sustainable product.

And we are equally proud to present this collection of the proceedings of the Application of Genome Science to Sustainable Aquaculture session at Aquaculture Canada℠ 09. We welcome the opportunity to explore new opportunities in this field.

Genome Atlantic is a not-for-profit organization with a mission to develop and lead a program of genomics research that delivers tangible economic, social and environmental benefits to Atlantic Canada.

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# Bulletin
## de l'Association aquacole du Canada
### 107-3

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## Application of Genome Science to Sustainable Aquaculture

**proceedings of a special session held at Aquaculture Canada$^{OM}$ 2009**

L.D. Hiemstra and W.S. Davidson, guest editors



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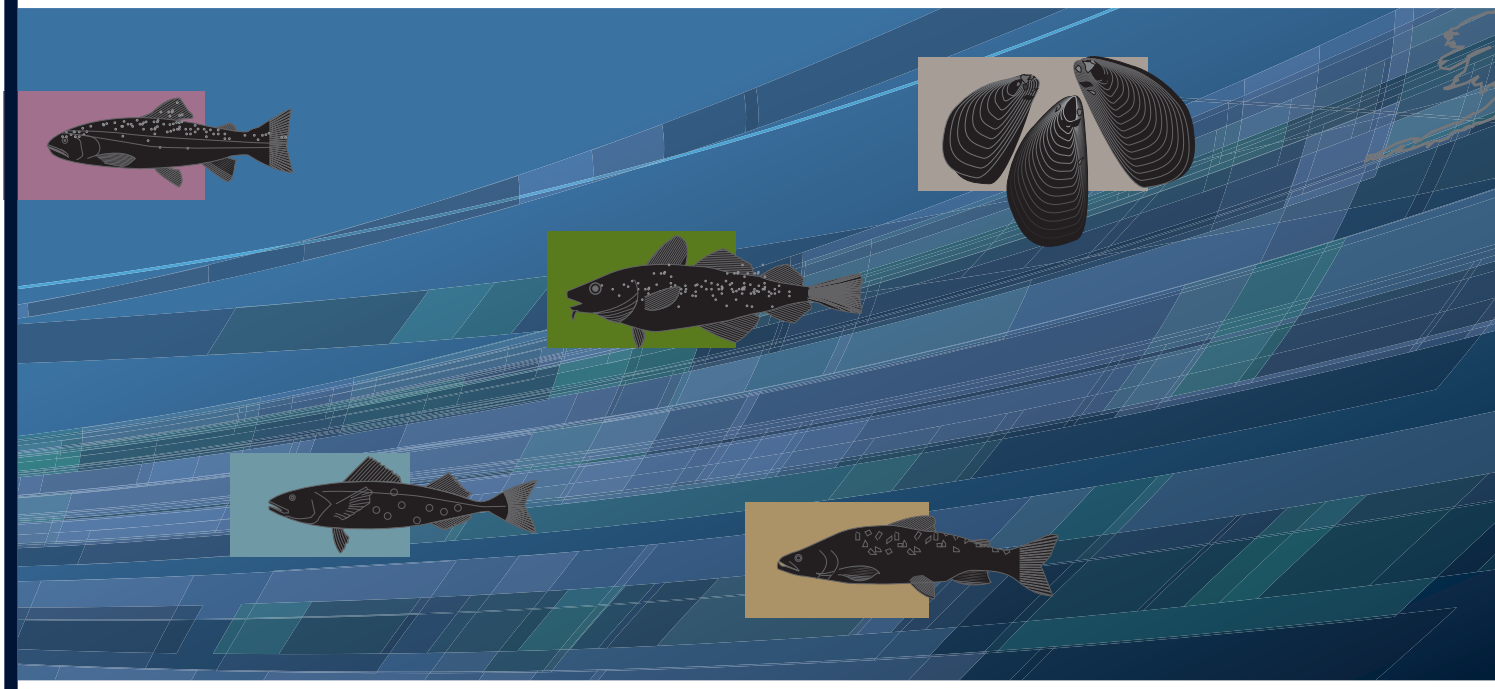
**Genome Atlantic** and **Genome British Columbia** provided financial support for the publication of these proceedings.


**Aquaculture Canada$^{OM}$ 2009,** the 26$^{th}$ annual meeting of the Aquaculture Association of Canada, was held at Nanaimo, BC, May 10-13, 2009. The theme of the conference—Aquaculture: Meeting the Challenges—provided an opportunity to emphasize the advances the industry has made and discuss opportunities for the future. The session, Application of Genome Science to Sustainable Aquaculture, was organized to highlight the contribution of genome research to successful aquaculture ventures. The session proceedings were prepared to illustrate the application of genome research to a wider audience.

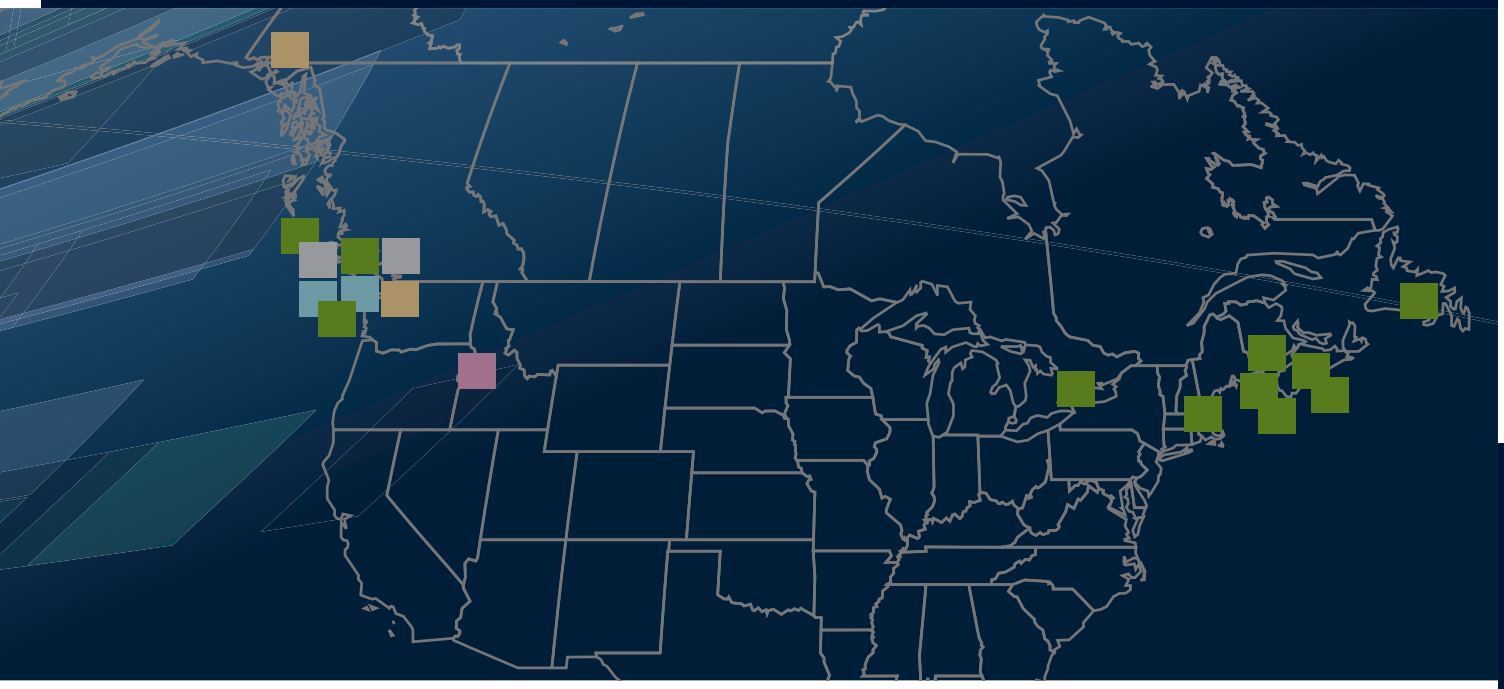

—**Linda D. Hiemstra** (session organizer and proceedings editor), Mel Mor Science, 6036 Breonna Drive, Nanaimo, BC V9V 1G1 and **William S. Davidson** (session chair and proceedings editor), Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC V5A 1S6

Application of Genome Science
to Sustainable Aquaculture

# Projects



| | | | |
|---|---|---|---|
| **Project Title:** | Research Thrusts in Nutritional Genomics of Rainbow Trout | Pilot Study of Sablefish Genomics | The Atlantic Cod Genomics and Broodstock Development Project |
| **Location:** | Hagerman Idaho | Victoria BC, Saltspring Island BC | St. John's NL, Halifax NS, Fredericton NB, Saint John NB, St. Andrews NB, Blacks Harbour NB, Portsmouth New Hampshire, Guelph ON, Vancouver BC, Nanaimo BC, Campbell River BC |
| **Species:** | Rainbow Trout | Sablefish | Atlantic Cod |



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|---|---|---|
| | The Myt-OME project: Development of a Health Assessment Tool for Marine Mussels | The Application of Salmonid Genomic Research in a Mid-Sized Commercial Broodstock Facility: The Icy Waters Arctic Charr Experience |
| | 2 research centres in Nanaimo BC | Vancouver BC, Whitehorse Yukon |
| | Mussels | Charr |

Commentary

# Genomics Science in Aquaculture: Providing Direct and Positive Effects on the Economy of Eastern Canada

**Brian Blanchard**

It is a great privilege to be part of a publication on the role of genomics science in Canadian aquaculture. I fully support aquaculture-related genomics research, as my personal experience with Genome Atlantic's Pleurogene Genomics Research Project has shown direct and positive effects for the industry.

The Pleurogene Project ENHANCING COMMERCIAL CULTURE OF ATLANTIC HALIBUT AND SENEGAL SOLE is a partnership between Genome Canada and Genome Spain, and local researchers from DFO–SABS, NRC–IMB and Scotian Halibut Limited. From 2004 to 2007, it explored the genomics of Senegal sole and Atlantic halibut. I am proud to say this project produced the world's first genetic linkage map of Atlantic halibut, giving Canadian producers a key tool for improving productivity and competitiveness by increasing our broodstock development capabilities.

In Canada, the priority is the creation of a science-based broodstock program aimed at reducing production costs and increasing quality and yield via the identification of commercially-relevant genetic traits such as disease resistance and rapid growth. Considering that the development of a halibut broodstock program requires 8 to 12 years per generation, information garnered via the Pleurogene Project is an invaluable contribution in terms of cost and time savings.

**Shelly LeBlanc with broodstock halibut**

The genetic map is a powerful tool that allows the industry to select superior halibut broodstock quickly and efficiently. We are able to use statistical approaches to identify genes of economic importance through quantitative trait loci (QTL) without having to actually

identify the gene. Broodstock selection can be guided with QTL markers correlated with traits of interest. Used in conjunction with family-effect information and traditional phenotypic election, we are seeing significant improvements in traits of economic importance. One of the immediate benefits is the ability to avoid inbreeding and poor-performing crosses.

We continue to work on trait heritability, family effect, QTLs and phenotypic responses, using the genomic map and the results of over 10 years of breeding with a fully-pedigreed Canadian broodstock. This effort has been combined with the dedication of students, staff and researchers from DFO, NRC, universities and industry partners and investors since the early 1990s.

Essentially, the research has reduced the guesswork and time that traditional breeding programs face, providing a decided advantage for the industry in the long term. Already, we have improved hatchery production and, as we move ahead, are confident that continued improvements in growout operations will be realized.

In closing, it is encouraging to see such a comprehensive research effort devoted to aquaculture. If the projections for the requirements of the industry are accurate—according to the Food and Agriculture Organization of the United Nations, production of 80.5 million tonnes per year will be needed—we have to continue this dedicated effort to improve and streamline our approaches to ensure a profitable and sustainable industry here in Canada and around the globe.

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**Juvenile halibut**

Introduction

# Moving from a World Leader in Aquaculture Genomics Research to Realizing Economic Benefits for Canada

**William S. Davidson**

The 21st century is being heralded as the era of genomics. The benefits of producing a sequence of the human genome and related resources are becoming evident with the advent of personalized medicine. The dream of the '$1,000 genome' will be realized in the next 10 years and within our lifetimes genomic analyses will become as routine as blood tests. But it is not just human health that is being impacted by genomic science. The genomes of approximately 40 other mammals have been or are being sequenced, and this comparative genomic information is being applied to selective breeding programs in agricultural animals—especially cows, sheep, pigs and poultry. On the other hand, fish present a huge challenge. With more than 20,000 species and an enormous diversity, deciding which species to choose for genomic sequencing was initially driven by the size of the genome (e.g., pufferfish have the smallest vertebrate genomes), followed by fish that are used as model organisms for development (e.g., zebrafish and medaka), and finally those selected for their ecological adaptations (e.g., stickleback). It is only recently that genomic resources for aquaculture species have been developed.

Canada has been at the forefront of aquaculture genomics and continues to be a leader in this field. This is due primarily to an influx of major funding through Genome Canada, Genome British Columbia and Genome Atlantic. The Genomics Research on Atlantic Salmon Project (GRASP) and its successor cGRASP (Consortium for Genomic Research on All Salmonids Project) have produced a myriad of genomic tools and resources for salmonids, including gene lists, genetic markers and microarrays for expression studies. As was described in the APPLICATION OF GENOME SCIENCE TO SUSTAINABLE AQUACULTURE SESSION at the Aquaculture Canada™ 2009 conference, these tools are being applied in breeding programs (e.g., in Arctic charr—see the paper on page 31 by McGowan et al.) and the assessment of alternative sources of feed (e.g., in rainbow trout—see the paper on page 11 by Hardy and Wacyk). GRASP and cGRASP have also been instrumental in the formation of an International Consortium to Sequence the Atlantic Salmon Genome (ICSASG).

With funding from Genome British Columbia, Norway and Chile, the Atlantic salmon genome sequence will act as a reference for other salmonid genomes. Indeed, the prospect of a salmonid reference genome motivated a French group to obtain funds to start sequencing the rainbow trout genome next year. It is quite realistic to expect that the genomes of Pacific salmon, trout and charr will be available within a decade. The Atlantic Cod Genomics and Broodstock Development Project (CGP) (see paper by Rise et al., page 21) is also a 'made in Canada' project that is funded by Genome Canada and Genome Atlantic. CGP has revitalized and revolutionized the cod aquaculture industry by producing genetic markers, genetic maps, and the tools for marker-assisted selection. The cod genome is being sequenced in Norway, and the resources developed through CGP will be instrumental in the annotation of the genome and translating this information into practical benefits for cod farmers in Canada and throughout the world. Genome Canada partnered with Genome España to fund the Pleurogene Project that produced the first genetic map for halibut, which is contributing to a selective breeding program for this species (see the commentary by Brian Blanchard on page 6).

The projects mentioned in the previous paragraph are all large-scale, both in terms of funding and scope. However, lesser amounts of money can still enable projects that will have a major impact on the aquaculture industry, especially when they are applied to emerging cultured species. The paper by Campbell and Koop (page 53) is particularly informative and shows how one can proceed very rapidly from essentially nothing being known about the genome of sablefish to having a gene list, a variety of genetic markers and a genetic map that can be used to identify quantitative trait loci (QTL) and thus be incorporated into a breeding program. It should be noted too that the genetic markers that are developed in genomics projects lend themselves to examining population structures and conservation genetics. Therefore, they benefit both aquaculture and wild fisheries.

The interaction of hosts and parasites is of great interest in all production systems. Genomics is tackling the controversy surrounding sea lice and their interaction with salmon through the GiLS (Genomics in Lice and Salmon) project funded by Genome BC. GiLS will examine how salmon react to sea lice—potentially providing the information to develop vaccines—as well as how sea lice react to salmon. In addition, this project will examine the population structure of sea lice and determine, for example, if there are different sea lice populations on different salmonid species.

Aquaculture is not restricted to finfish, and this is also true when it come to genomics. Shellfish, particularly mussels (*Mytilus* sp.) are valuable aquaculture species in British Columbia and eastern Canada. However, mussels are also recognized as an important bioindicator of environmental health. The *Myt*-OME Project (see paper by Gurney-Smith and Johnson, page 45) will identify genes involved in environmental stress responses in *Mytilus* sp. The resources that will be produced as part of this project, such as an expression microarray, will thus benefit both aquaculture managers, who will be alerted to when their shellfish may becoming stressed, and

also agencies whose mandate is to monitor the health of the marine environment.

As indicated above, Canada was able to become a world leader in aquaculture genomics when Genome Canada and its partner genome centres, Genome British Columbia and Genome Atlantic, received major funding. It should be noted that unlike the Canadian federal granting agencies NSERC and CIHR, Genome Canada does not have an annual budget and has relied for its funding on government year-end surpluses (obviously a thing of the past in the current economic climate) and its ability to persuade politicians that genome science should be funded at a competitive level in this country. In addition, a new model of funding was established that provided a maximum of 50% of the cost of a project. The remainder has to be obtained through partnerships (e.g., with industry, or through international collaborations). The lack of new funding for Genome Canada in the 2009 federal budget has seriously damaged the sustainability of genome science, particularly when it comes to aquaculture genomics and animal production in general. Some of the slack has been taken up by smaller programs initiated by Genome British Columbia with the aid of provincial funding, and this is very welcome news for researchers in that province. But Canada is in danger of losing its leadership role in aquaculture genomics just when the translational aspects of several established projects are emerging. Having made a prior commitment to research, Canada will not realize the economic and social benefits if research support is not continued. Therefore, our political leaders must be made aware of this impending problem and persuaded that the return on their investment is indeed worthwhile when it comes to genome science.

In addition, there is a need for industry to embrace the new genomic technologies and form partnerships with the academic groups that have built up expertise in terms of trained individuals as well as the application tools. The technologies developed in collaboration with industry will support enhanced management and culture practices leading to greater industry stability and growth with wide-reaching economic benefits for Canada.

The purpose of the Application of Genome Science to Sustainable Aquaculture Session at Aquaculture Canada$^{OM}$ 2009 was to showcase current research projects and how this research applies to aquaculture genomics in this country. The success of this inaugural session will be measured by the partnerships that are formed and the collaborative projects that take place in the next couple of years. All who were involved in this session, either as speakers or as attendees, should strive to maintain the dialogue and seek ways to better understand one another's needs and capabilities.

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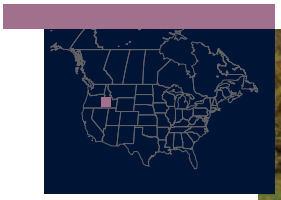
# Research Thrusts in Nutritional Genomics of Rainbow Trout



Ron Hardy

**Ronald W. Hardy and Jurij Wacyk**

Sustainable aquaculture depends in part on the use of sustainable inputs and on minimizing outputs that affect the aquatic environment. Challenges facing the aquaculture industry include the fact that fish meal and fish oil are finite resources that are bound to increase in cost as demand by the aquafeed industry taxes world supplies. Using alternative ingredients derived from grains and oilseeds is widespread, but replacing more than approximately half the fish meal or fish oil in feeds for salmonids and other marine piscivorous species is difficult. Genomic tools are being applied to this problem in a number of ways, ranging from metabolic responses to alternative proteins, to investigations on whether differences in omega-3 fatty acid retention exist among strains or family lines of fish. Dietary inputs that affect muscle cell formation, long-term responses to dietary carbohydrate levels, and factors affecting mineralization of bone are all being revealed through the application of genomics in aquaculture research. Basic research to understand protein retention as well as protein turnover will lead to a reduction in waste outputs from fish farms and to more precise diet formulations containing alternative protein and oil sources.



Nutritional genomics, or nutrigenomics, is an emerging field in human and animal nutritional research holding great potential for studying fish nutrition. Nutrigenomics—using molecular and genomic techniques—aims to determine the effects of dietary ingredients on the genome (i.e., gene expression) to understand different aspects of nutritional biochemistry, metabolism and physiology.[1] Put another way, nutrigenomics studies the interactions between the diet and the genome to reveal the effects of nutrient intake on genetic responses.

Gene expression, along with other mechanisms, presumably controls cellular metabolism, and cellular metabolism presumably controls fish physiology, meaning growth, health, behavior, reproduction, and everything else fish do. In practical terms, genomics has the potential to increase our understanding of the factors controlling muscle growth, energy partitioning, fatty acid and protein metabolism, and immune responses as well.

**"... genomics has the potential to increase our understanding of the factors controlling muscle growth, energy partitioning, fatty acid and protein metabolism, and immune responses as well."**

Genomics gives us a new way to examine how and why changes in feed formulation, feeding rate, levels or forms of nutrients in feeds and so on, affect fish performance, the economics of aquaculture, retention and loss of nutrients by fish, and the quality of farmed fish products to the consumer. For the most part, nutrigenomics in aquaculture is focused on aspects of fish production influenced or controlled by nutrition and feeds. Information learned from nutrigenomic research in fish can help identify targets for nutritional manipulation or modifica-

tion to control such things as muscle fiber number and hence fillet texture, protein accretion and turnover. Nutrigenomic research can help in the development of strategies for phase-feeding diets containing plant oils and fish oils to ensure healthful levels of omega-3 fatty acids in fillets, or tell us if immune function is affected by feeding plant protein concentrates or plant oils, probiotics or immune stimulants. In short, nutrigenomics offers much promise in fish nutrition research and, providing it is used wisely, can help us find optimal combinations of dietary ingredients to maximize fish growth performance.

The potential of nutrigenomics is widely recognized, but only relatively recently have papers appeared in the scientific literature utilizing nutrigenomics in fish nutrition research. One factor that has contributed to recent progress is the sequencing of genomes of humans, mice and zebrafish, plus progress on sequencing other species of fish. This advancement has generated constantly growing and more complex genomic data sets, that combined with progress in disciplines like biochemistry, genetics and bioinformatics, have provided nutrition researchers, including fish nutritionists, with the necessary tools to study interactions between diet composition and an organism genome.[2]

Nutrigenomics has also been described as a discipline at the interface between nutrition, genetics, molecular biology and medicine due to its search of links between different phenotypes generated by the diet and changes in genetic responses.[1] To accomplish this task, nutrigenomics uses high throughput techniques to look at different levels of the process between the genome and the phenotypic response of an organism. Sequencing and gene expression techniques like DNA chips or microarrays as well as real-time PCR (polymerase chain reaction) lie at the base of studying gene-diet interactions.[3]

In brief terms, sequencing can be defined as the determination of the nucleotide order of a DNA/RNA fragment. This technique is based in the methodology developed by the Nobel laureate Frederick Sanger. The automation of the Sanger sequencing has generated a wealth of genome data that have paved the way for the development and use of other molecular techniques.[2,4]

Among these techniques, DNA chips or microarrays have helped accelerate the study of gene expression changes associated with different conditions and treatments due to their capacity to measure simultaneously the expression of up to ~20,000 genes, like the cGRASP chip for salmonids.[5] More focused approaches can also be used to measure gene expression using smaller sets of genes associated with particular metabolic pathways or physiological responses, such as muscle fiber number, amino acid accretion and degradation (turn-over), dietary energy partitioning (i.e., glycogen and fat deposition), among other biologically important processes.

Even when microarray technology permits genome-wide analysis under specific sets of conditions, the specific features of this technology means that the results must be verified using more sensitive tools.[6,7] For this purpose rt-PCR is considered the gold standard. This technique, based in the reaction catalyzed by the enzyme polymerase, was basically re-discovered in the 1980s by Kary Mullis who used the bacterium *Thermus aquaticus* polymerase to avoid the destruction of the enzyme by the high temperatures used for the polymerization process. This technique is nowadays routinely used for genomic research all over the world.

These technologies (microarray and rt-PCR) rely on gene sequence information to measure the amount of messenger RNA transcribed/produced when a specific gene is expressed as a result of a pre-determined set of conditions or treatments. More technical details about microarrays and rt-PCR can be found in reviews by

Jaluria et al., Bustin et al., Valasek and Repa, and Breitling.[7-10]

Early work in the area of gene expression in fish nutrition studies was not well focused. It consisted of subjecting fish to dietary treatments and looking for genes that were up- or down-regulated. While this was valuable in that it demonstrated the responsiveness of fish genes to dietary changes, it did not provide insight into how metabolism is regulated or how metabolism can be altered by intake of specific nutrients or compounds found in feed ingredients. More recently, the field has matured and genomics is being used in a more targeted, hypothesis-driven fashion and genes being examined for expression levels are those having regulatory or rate-limiting roles in specific metabolic pathways. This more focused version of genomics has been called functional genomics.[1]

The history of nutritional research, mainly in livestock, laboratory animals and humans, has gone through many phases characterized by changes in the theoretical assumptions and technologies driving research over a given period of time. Even when these changes occur at different rates, some time is required for certain concepts that we accept without question today to become accepted by the research community and society.[11] Historical records show the recognition of a connection between nutrition and disease is very old; an example is night-blindness in horses that could be cured by feeding forages rich in beta-carotene, a precursor to vitamin A. Nevertheless, a century ago, the concept that lack of a specific nutrient could be the cause of specific diseases was not widely accepted.[12] Food was considered fuel to power the body. It was only during the 20$^{th}$ century that links between specific nutrients and specific diseases were made—such as the link between rickets and vitamin D intake or between beriberi and thiamin intake. A key factor that led to acceptance of the concept of dietary requirements for essential nutrients was that deficiencies of specific, individual nutrients caused diseases of discrete body systems in a relatively short period of time, and these conditions could be cured rapidly by supplying the essential nutrient. Had deficiency diseases not had a short latency period or not resulted in abnormalities of discrete body systems (e.g., bones or vision), it is unlikely that our concept of minimum dietary requirements would have been developed. For example, the minimum requirement for vitamin D was established as the intake level needed to prevent rickets and the minimum requirement for vitamin C was established as the intake level needed to prevent scurvy. More recently, nutritional science supported by genomic studies, has expanded beyond the single nutrient–single short-latency disease concept to embrace the idea that nutrient deficiencies can cause long-latency diseases that are different from short-latency diseases or abnormalities, and can affect a wide range of tissues or systems, thereby influencing system biology.

Nutrients influence metabolic pathways through mechanisms ranging from providing essential substrates for metabolic reactions, to causing a cascade of events leading to widespread changes in gene expression. Thus, essential nutrients are essential not only to prevent discrete diseases in specific tissues, but also to support metabolism in virtually all cells in the body. This is well illustrated by the connections between vitamin D intake and

risk of multiple disorders in humans such as Type 1 diabetes, hypertension, various cancers, multiple sclerosis, and periodontal disease. The dietary vitamin D intake levels required to lower risk of these and other disorders are much higher than those required to prevent rickets in children. This brings into question the concept of minimum dietary requirements based upon prevention of discrete diseases, and has implications for fish nutrition research.

Advances in human nutrigenomics have been valuable to fish nutrition research, but the issues challenging the aquafeed industry are less related to short- or long-latency deficiency diseases associated with specific nutrients and more related to replacing fish meal and fish oil with plant proteins and plant oils, making more efficient use of dietary nutrients, improving product quality, fish health and welfare, as well as aquaculture sustainability.

However, the concept of minimum dietary requirements for essential nutrients in aquafeeds requires more study, because genomic studies suggest that some nutrients, such as amino acids, can drive metabolism in unexpected and sometimes undesirable ways. Formulating aquafeeds to ensure that levels of all essential amino acids are at or above minimum dietary requirements is important, but this alone may not be sufficient to ensure optimum growth or protein retention in fish. Mammalian studies have shown that different protein sources have differential effects on protein retention. The effect of the protein source on protein retention appears to be associated with the speed at which dietary amino acids are absorbed after digestion, making them more or less rapidly available for metabolic purposes, a response that appears to persist beyond the end of the meal. This effect has been linked to a differential effect of protein sources to stimulate protein synthesis and inhibit protein breakdown in mammals. Rapidly absorbed amino acids—despite stimulating greater protein synthesis than do slower absorbed ones—also stimulate greater amino acid oxidation, generating lower protein retentions.[13-15]

Also, the ratios of essential amino acids to each other and the ratio of nonessential to essential amino acids in feeds are being found to be important determinates of efficient retention of dietary protein as tissue protein in fish. Green et al.[16-18] found that the amino acid profile suggested for rainbow trout was the closest to a dietary optimum for rainbow trout in terms of maximizing nitrogen retention and minimizing its excretion.[19] They also found that the proportion between indispensable and dispensable amino acids played an important role in nutrient utilization, suggesting a dietary proportion ranging from 0.96 to 1.63 would maximize nitrogen retention from dietary protein sources.

Similarly, differences possibly related to the structural forms of proteins in fish meal compared to those in plant protein concentrates are being found to influence gene expression, leading to shifts in cellular metabolism, protein synthesis and degradation, and lipogenesis. The detrimental effects of high levels of inclusion of plant protein sources in carnivorous fish diets have been linked with modifications in protein metabolism pathways. Part of the detrimental effects of increasing inclusion of plant protein products (soy protein concentrate, soybean meal and wheat gluten) are attributed to a decrease in the activity of enzymes like alanine and aspartate aminotransferase in the liver of Atlantic cod.[20]

Wacyk and Hardy (unpublished data), working with rainbow trout fed amino acid balanced diets using fish meal (FM) or soy protein isolate (SPI) as the main protein sources, observed a significant reduction in growth performance when fish were fed the SPI diets. This growth depression was associated with changes at the level of transaminase reactions. Significant decreases in the transcription of alanine amino tranferase and glutamine synthetase were observed for fish fed

SPI-diets when compared to fish fed FM diets while the expression of genes like asparate amino transferase, asparagine synthetase and glutamate dehydrogenase was reduced. Evidence for this type of response has also been found in mammals. For example a strong decreasing effect of SPI in the hepatic activities of alanine and aspartate aminotransferase was observed when compared to casein-fed rats.[21] Studies in mammals have shown adaptive type responses to high protein loads up-regulating amino acid metabolizing enzymes.[22] However when this capacity is overwhelmed, down regulation of this pathway has also been observed. The explanation for this appears to be related to a metabolic reaction to protect the liver and to ensure its catabolic capacities are not exceeded, but there is no consensus about this response.[22]

In mammals the mTOR (mammalian target of rapamycin) cascade has been described as an integrator of cellular status of which energy levels, growth factors, nutrients, and stress levels are able to signal their status to this pathway through a complex network of proteins. Two branches have been described so far for this pathway, one more directly involved with cell growth and a second one that has been described as nutrient sensitive. In mammals one of the signals that has been shown to modulate the activity of the nutrient responsive part of the cascade is amino acids, especially branched chain amino acids, but the way their abundance is transmitted to the mTOR cascade is far from clear.[23-25]

While recently working with rainbow trout, mammalian-type responses were reported on the TOR cascade in term of responsiveness to nutrient intake.[26] Working with a trout muscle cell line, they were able to show the level of amino acids was able to regulate TOR signaling as measured by changes in the phosphorylation status of TOR, PKB, p70 S6 and eIF4E-BP1, all molecules used in mammals as indicators of varying protein synthesis activity.[27] In our laboratory, studying transcriptional changes associated with the TOR cascade, we found the expression levels of TOR and REDD-1 were also differentially affected by feeding the fish SPI or fishmeal based diets. While gradual decreases in the expression of TOR were observed for both treatments, the fish fed the SPI showed significantly lower levels of TOR expression. On the other hand REDD-1 showed significantly higher levels of expression throughout the duration of the study in fish fed SPI-based diets. In mammals, the expression of REDD-1 has been shown to be up-regulated under a variety of conditions related to cellular stress. In a recent report using human cell lines,[28] a proposed model for REDD-1 regulation in which a cascade of events apparently initiated by endoplasmic reticulum (ER) stress, trigger REDD-1 transcription. The authors also postulated that this elevation of REDD-1 transcription may be related to a negative feedback mechanism to reduce mTOR signaling to attenuate ER stress. ER stress responses have also been described in fish but under a different set of conditions. Researchers working with zebrafish and rainbow trout cell lines reported a decrease in protein synthesis in response to both ER stress and to infectious pancreatic necrosis virus infection, suggesting that the ER stress related response is well conserved in fish as in other higher eukaryotes.[29]

The most active area in which genomics is revealing the effects of dietary nutrients on cellular metabolism in fish is associated with glucose metabolism. Starch is the least expensive source of dietary energy on the planet, and gelatinized starch supplies glucose in aquafeeds, plus acts as a nutritional binder. Researchers and aquafeed producers want to know several things about starch and other carbohydrates in plant-derived feed ingredients. First, what is the optimum level of gelatinized starch in the diet to provide energy to spare protein for growth? Second, what is the maximum level of gelatinized starch (or glucose) that carnivo-

rous fish species can tolerate? Third, do non-starch, non-soluble polysaccharides present in grain or oilseed protein concentrates contribute energy to aquafeeds, or do these compounds interfere with digestion or otherwise alter metabolism?

Research addressing these questions is underway in several laboratories around the world. In a study comparing the responses of zebrafish, a species for which the entire genome has been sequenced, and rainbow trout to varying levels of dietary glucose supplied by gelatinized starch supplemented from 0% to 35% into experimental diets, the glycolytic responses of the two species were found to be similar. Physiological responses, such as plasma glucose levels and liver glycogen levels, were highly correlated with expression levels of key enzymes involved in glucose metabolism, and highly correlated to dietary starch level. Zebrafish mature at an early age, and maturation was found to affect gene expression differently in male and female zebrafish.[30,31] Trout exhibited a capacity to tolerate the highest level of starch by shifting metabolism towards glycogenesis, but this capacity was limited by upper limits on glycogen storage in liver and muscle (Powell et al., unpubl.). Once glycogen stores reached saturation, fish showed signs of metabolic stress, suggesting that health and performance would be affected by prolonged feeding.

Expression of the gene for glucokinase, the first enzyme in the pathway by which glucose is metabolized in cells and the rate-limiting enzyme in this pathway, was induced by blood glucose levels, with a maximum induction at 6 mM blood glucose. Taking together both physiological data and gene expression information, it appears that about 15-17% available starch in trout diets is optimal, although fish can tolerate higher levels without affecting growth. However, the capacity to tolerate high amounts of available starch is limited, as shown by expression levels of genes associated with REDOX potential. In diabetes research, chronic hyperglycemia has been linked to the generation of oxidative stress in different organs including the liver by mechanisms that are not well understood.[32-34] Carnivorous fish have been considered to show diabetes-like responses in relation to intake of dietary carbohydrates, illustrated by prolonged hyperglycemia after a carbohydrate-rich meal. Evidence from studies in our laboratory indicates that rainbow trout can adapt to diets with digestible carbohydrates up to 35%. This adaptation seems to be related to changes in hepatic transcription of genes involved in transamination reactions as well as genes related to the REDOX environment in the liver. Changes in expression for the first group of genes appear to be part of the adaptations required to use carbohydrates as energy sources to spare amino acids, as reflected by the higher protein retention efficiency observed for fish fed diets with up to 15% of dietary CHO. However, higher dietary inclusion of CHO in rainbow trout (>15%) seemed to overcome the fish capacity to adapt. This is likely associated with changes in expression levels of REDOX genes as suggested by lower protein retention observed for these treatments along with the higher levels of whole body fat deposition (Wacyk and Hardy, unpublished data).

Research on basic physiological and genomic responses of fish to dietary glucose or carbohydrate level is useful in the context of developing sustainable feeds for salmonids using protein concentrates or rendered animal proteins to replace fish meal. Plant protein concentrates are very different from fish meal in a number of ways. First, amino acid profiles differ between plant proteins and fishmeal proteins. Fishmeal amino acid profiles are ideal for farmed fish, while plant proteins are all deficient in one or more essential amino acids. Second, some alternate proteins have high levels of branched chain amino acids, such as corn gluten meal (leucine) or blood meal (isoleucine). Third, plant protein concentrates lack certain nutrients that may be essential at early life-history stages in fish, such as

taurine. Fourth, unlike fish meal, plant protein concentrates do not contain androgens or bone minerals. Fifth, plant proteins contain phytoestrogens, phytic acid and antinutrients. Finally, as mentioned earlier, fish meal contains structural proteins, like muscle, that takes some time to digest, whereas plant proteins are hydrolyzed to amino acids and absorbed in the digestive tract relatively quickly. Some amino acids and their metabolites may have signaling roles in metabolism, driving protein synthesis and/or degradation or possibly energy-yielding pathways.[35] Feeds based entirely on plant protein concentrates can be formulated to meet all essential amino acid requirements of rainbow trout without difficulty, and trout grow quite well using these feeds. But trout never grow quite as well as they do when fed fishmeal-based feeds, and protein retention ratios (protein gain/protein fed) are always lower in fish fed plant protein concentrate feeds compared to fishmeal-based feeds. Metabolic changes associated with high levels of plant protein sources have been described to affect metabolic routes in the liver of rainbow trout. Using a combination of maize gluten meal, extruded peas and rapeseed meal to replace fish meal in rainbow trout diets, poor performance of the fish fed the plant meal diet was observed.[36] They concluded this response was associated with significant alterations in the level of proteins involved in energy generation, maintenance of reducing potential and cellular protein degradation. They also observed that plant protein diets induced increases in the generation of REDOX power in the liver. This was concluded considering an elevation in transaldolase and malate dehydrogenase protein levels associated with increasing activity of glucose-6-phosphate dehydrogenase in the plant-based diets, which may have been affecting lipid production. Along the same line, glucose-6-phosphate dehydrogenase transcription was observed to be up-regulated in the fish fed the SPI diet when compared to fish fed the FM-based diet (Wacyk and Hardy, unpublished data). Elevated transcription of this enzyme is probably part of the mechanism behind energy partition in the liver of rainbow trout—in this case towards an increase in fat deposits. Evaluations for performance and liver proteome changes were made for rainbow trout that were fed two diets with high inclusion levels of plant protein but differing in their soybean meal content (SBM).[37] They found that even when growth rates were not altered by the dietary treatments, significantly lower protein efficiency utilization, higher hepatic transaminase activity and higher hepatic levels of proteins related with stress response were observed for the diet with increased SBM levels

How can genomics help us understand these differences and where do we go from here? Several studies[30,31,36,37] and work recently completed in our laboratory have increased our understanding on how common dietary components are able to modify gene expression and finally the phenotype of rainbow trout. As mentioned above, the objective of nutrigenomics is to study the effects of the dietary components on the genome, but gene expression is one of the layers of information necessary to understand nutritional factors in fish metabolism. One must keep in mind that gene transcription is only part of the complex mechanism by which metabolic pathways are regulated[38] and the fact that the genes under study responded to the experimental treatments does not necessarily imply that other regulatory points will respond in a similar way.[39]

An important aspect to consider is that fish are fed mixed meals made of various nutrients including lipids, carbohydrates, protein, minerals, vitamins and other nutritive and non-nutritive feed additives.[40] This means that postprandial effects of the diets over fish metabolism after digestion and absorption of nutrients is a complex process that involves numerous potential interactions.[41] Considering

that rainbow trout use carbon skeletons from amino acids for energy purposes,[40] interactions between energy sources of the diet (carbohydrates, lipids and proteins) and the modulation of these interactions by the postprandial effect of the diets are also an important factor influencing nutrient use.[40,41]

Having the above mentioned factors in mind, the use of more integrative approaches, simultaneously using other genomic tools to study changes in protein levels and activities (proteomics) and the characterization of metabolic fluxes (metabolomics) will provide a more complete picture of the effects of the diet in fish metabolism. Such integrative approaches have been coined system biology, which in mammalian research is transforming the traditional reductionist approach of studying the effect of a single nutrient over specific genes into a holistic one.[42]

Although fish nutrition research is not quite at the same level as mammalian nutrition research, the mammalian paradigm can be a useful road map to rapidly move towards more integrative approaches. Genomic tools currently being used by fish nutritionists will continue to aid in achieving this goal. For example, the availability of glucose and amino acids plays an important role in gene transcription through complex mechanisms involving the interaction of transcription factors as well as characteristic sequence elements of each gene. These sequence elements correspond to short fragments of DNA (i.e., 6 to 12 bp) that contain specific nucleotide sequences that appear to confer transcriptional tissue specificity as well as the ability to respond to hormones and nutrients.[43] From studies with mammals, glucose responsive sequences have been identified in genes like hepatic pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) genes that, if we consider their function, also show a link between carbohydrate and lipid metabolism. Also in mammalian cells, mRNA levels of amino acid transporters (CAT1, ATA2), transcription factors and genes related to protein metabolism (ASNS, CHOP, IGFBP-1) and ribosomal proteins (L17, S25, L35) have shown to increase according to amino acid deprivation in culture media, a response that seems to be associated with nutrient/amino acid response elements in these gene sequences as well.[39] The identification of these types of response elements in genes that show significant changes in their transcription in fish as a response to diet represents an interesting area of research that will lead to better understanding the mechanisms behind altered gene expression. Identification of this type of sequence can also help in marker-assisted selection programs if differences in the sequences of these response elements are correlated with improved fish performance.

Studying other regulatory points in metabolic pathway regulation will also help us better understand changes associated with the replacement of FM in rainbow trout diets. Evaluating the activity of the enzymes coded by the transaminase genes and the genes related to the REDOX environment of the liver will provide a more complete picture of the effects of increasing levels of dietary carbohydrates as well as changing the protein source of the diet in order to find out if transcriptional changes correspond with changes in the associated enzyme activities.

In the case of genes like TOR the evaluation of the activation of kinase activity of components of this cascade have already shown mammalian type responses in rainbow trout[26] which, in association with transcriptional changes in genes like REDD-1, will help reveal the role of this cascade in protein metabolism in fish and how the replacement of FM as a protein source will affect it. Evidence from mammalian research will also assist in this objective. For example, in a recent study in rats fed complete diets, significant correlations were found between muscle protein synthesis and plasma leucine levels and with the activation of S6K and 4E-BP1 up to 90 minutes after a meal. S6K1 was the first component of the mTOR

pathway to be identified and along with 4E-BP1 phosphorylation states has been used to measure the activity of the nutrient responsive branch of the mTOR cascade.[44] This evidence indicates that leucine may act as an acute stimulator of muscle protein synthesis through the activation of the mTOR pathway as observed by the activation of S6K and 4E-BP1. This study also shows that the leucine content of the diet is able to acutely activate muscle protein synthesis but not its duration after a meal, highlighting the importance of protein/leucine intake during the day as a gfactor to optimize anabolic responses.[45] The evaluation of this type of responses of the mTOR cascade will help assess the effects of FM replacement in rainbow trout diets and its effects on protein synthesis.

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# The Atlantic Cod Genomics and Broodstock Development Project

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The objective of the Atlantic Cod Genomics and Broodstock Development Project (CGP) is to develop breeding programs and fundamental genomics tools which will be used to supply the developing Atlantic cod aquaculture industry in Canada with improved broodstock (www.codgene.ca). The first three major spawning seasons have been completed, including rearing of fish in hatcheries through to transfer of juveniles to seacages. The fourth spawning season, which was the first to include elite broodstock as parent fish, is also completed. Evaluations of progeny have included assessment of family performance related to growth and overall health. In addition, investigations related to cod physiology and immunology have been conducted. Considerable variations among families have been observed. Heritability estimates suggest that the breeding programs will yield improved performance. The CGP has dramatically improved availability of genomic resources for cod. Approximately 158,000 sequences have been submitted to GenBank. A 20,000 element (20K) oligonucleotide microarray is being printed and tested. Development of gene-linked markers and a high density genetic map are ongoing. Marker identification has yielded > 4,500 "predicted informative" single nucleotide polymorphisms (SNPs) and >140 microsatellite markers. In addition, social scientists have worked closely with other CGP scientists and industry partners to examine ethical, environmental, economic, legal and social issues related to the CGP. Resources developed by the CGP will enable marker assisted selection, and provide valuable tools for Atlantic cod research.



## Project Overview

Declining wild populations of Atlantic cod worldwide have resulted in renewed interest in farming this species. This is particularly true in Atlantic Canada where,

for many years, the fishing industry has been a major contributor to the region's cultural identity and economy. Reduced catches have brought many challenges to the fishing industry and to those dependent on its revenue. Growth of the salmon aquaculture industry has helped to revitalize the local economy in some areas, but the aquaculture industry in the Atlantic Region would benefit from diversification to other species to sustain growth.

There are several reasons for initiating Atlantic cod *(Gadus morhua)* (Figure 1) farming today and for strengthening ongoing efforts related to the following: a) the existence of a broad spectrum of scientific knowledge; b) investments by private industry in closed production life cycles, and engagement through to marketing; c) contributions by Federal and Provincial governments towards both infrastructure and support; d) unpredictable and seasonally constrained wild fish landings of gadoids including cod; and e) the observation that most wild cod stocks are producing far below maximum sustainable yield.[1] It is noteworthy, however, that cod stock status varies, ranging from collapsed (Newfoundland cod of the Grand Banks) to seriously over exploited (North Sea and Baltic cod), while the status of other stocks is considered moderately well (Icelandic cod) or satisfactory (northeast Arctic cod).[2] Faced with dwindling stocks and fluctuating market value of farmed Atlantic salmon (*Salmo salar*), several countries have launched large projects to expand cod culture significantly.

The Atlantic Cod Genomics and Broodstock Development Project (CGP) is an $18.1 million project that is funded in part by Genome Canada, Genome Atlantic and the Atlantic Canada Opportunities Agency. The objective of the CGP is to develop a breeding program and a set of fundamental genomics tools which will be used to supply the developing Atlantic cod aquaculture industry in Canada with improved broodstock. The CGP has established family-based breeding programs in Newfoundland and New Brunswick/New Hampshire. The first three major spawning seasons have been completed, including rearing of fish in hatcheries through to transfer of juveniles to sea cages. In addition, in Newfoundland, land-based broodstock are also maintained. Following several assessments of performance, elite broodstock have been selected to retain as parents for future year classes. The fourth spawning season, which represents the first major spawning season to include elite broodstock as parent fish, commenced in the fall of 2008 and the progeny were reared at the Dr. Joe Brown Aquatic Research Building (JBARB) in the Ocean Sciences Centre, Memorial University of Newfoundland, and as well as at the newly constructed hatchery at the Huntsman Ma-

**Figure 1**
***Gadus morhua* raised in captivity as part of the CGP objectives.**

rine Science Centre in St. Andrews, NB. The establishment of family-based selective breeding programs in New Brunswick and Newfoundland and Labrador is ensuring that local stocks can be used for the benefit of the provincial industries.

The CGP transitioned from using only wild-caught broodstock to utilizing selected broodstock during spawning in 2008. In fall of 2009 the CGP team will be completing a second round of spawning using broodstock selected for improved performance traits. Traditionally, Atlantic cod aquaculture has depended on unselected wild cod stocks for production which has limited progress for the industry. The CGP's captive selective breeding program is a first step towards domestication of this species. At the end of the project, ownership of elite cod broodstock developed by the CGP will be transferred to the founding industry partners in New Brunswick (Cooke Aquaculture Incorporated) and Newfoundland and Labrador (Northern Cod Ventures and Newfoundland Cod Broodstock Company). These will represent the first domesticated broodstock for cod aquaculture in Canada. The CGP's Collaborative Research Agreement (CRA) includes a non-breeding clause that serves to block breeding of CGP developed fish by competitors, ensuring a lasting benefit to Atlantic Canada and a sustainable competitive advantage for the founding industry partners. Valuable traits for the aquaculture industry, such as growth, health, sexual maturation, stress tolerance, fillet quality and yield, are being measured and evaluated. We are also determining the feasibility of incorporating specific traits in future breeding programs to ensure fast growing, healthy, high quality Atlantic cod.

In parallel to the activities within the breeding program, the CGP has sequenced thousands of cod genes and developed other tools to support the selective breeding program as well as other research activities on Atlantic cod. Using the sequence information generated through the CGP, cod gene sequences have been examined for differences among individual fish. These differences (molecular markers) will be associated with fish that perform well or poorly under aquaculture conditions. Once the associations are known, the markers can be used to non-lethally screen potential broodstock for traits of interest. Resources developed by the CGP will be used to identify variation in Canadian broodstock, to develop gene-linked markers for use in broodstock management, and for comparative genomics. Further, the CGP has developed a number of tools that enable the study of cod gene expression. These tools are being used to examine how Atlantic cod respond to stressful conditions and exposure to pathogens. Prior to the CGP, a major factor limiting the directed improvement of cod broodstock had been the scarcity of molecular tools available for cod. The CGP has dramatically increased the genomic resources available for this species.

### Broodstock Development

The CGP has made significant advancements in the family-based breeding programs in Newfoundland and Labrador and New Brunswick/New Hampshire. The first three major spawning seasons, resulting in the generation of Year Classes 1 (2006), 2 (2007) and 3 (2008), have been completed at both locations. A total of 345 families from three year classes were available for evaluation by fall 2008. From these families, just over 137,000 fish were transferred to sea cages in the Atlantic region to enable the CGP to monitor their growth and performance in a commercial-scale setting by November 2008. During the project, once family fish reach an average weight of 15 grams, they are PIT tagged and assessed (Figure 2). PIT tags, or Passive Integrated Transponder tags, are used to mark and identify individual fish. Throughout the spring and summer, staff track family performance

related to growth, survival and the overall health of the progeny. Significant variation in growth between the families has been observed, and preliminary heritability estimates have been calculated. These data suggest that the breeding programs have the potential to be successful at improving growth rates for aquaculture through selective breeding. In addition to fish being evaluated as part of the CGP experiments, nearly 190,000 surplus cod were available for industry partners and collaborative research.

Rearing of Year Class 4 has also been completed in Newfoundland and Labrador and New Brunswick. In NL, spawning was completed as part of a Fisheries and Oceans Canada Aquaculture Collaborative Research and Development Program-funded project in Newfoundland. At the JBARB, spawning employs photoperiod manipulation to advance the spawning cycle six months ahead of ambient spawning (which normally occurs in spring). Meeting this goal has demonstrated the ability to spawn cod in captivity in a manner that allows for juvenile growth over winter and a spring entry into sea cages. This satisfies the needs of industry in Newfoundland as it will ensure optimal growth of stocked cod over summer months. In New Brunswick, top-performing individuals from Year Class 0 (2005) and Year Class 1 (2006) that were stocked in Cooke Aquaculture sea cages in 2005 were transferred to the Huntsman Marine Science Centre to be used as parents in the generation of New Brunswick/New Hampshire Year Class 3 (2008) and 4 (2009) juveniles. Year Class 0 fish were reared as part of a Fisheries and Oceans Canada Aquaculture Collaborative Research and Development Program-funded project in NB. After the cod are transferred to sea cages, fish are

**Figure 2**
**Cod rearing in the CGP showing (left to right) cod larva, young juveniles in a first feeding tank, ~15 g juveniles, PIT tagging, transport to seacages, transfer into seacages and seacages. CGP cod were reared in aquaculture facilities for approximately one year prior to transfer to seacages where they were reared for an additional two years prior to harvest evaluation.**

evaluated for performance on an annual basis. The data are analyzed and used to calculate estimated breeding values (EBV) which guide the selection of elite broodstock. Spawning cod that were selected based on their breeding value is an important milestone achievement, and plans are in place to expand on elite broodstock development as project fish reach maturity.

In New Brunswick, research related to spawning includes the advancement of techniques for cryopreservation of cod sperm. The New Brunswick team is taking advantage of computer assisted sperm motility analysis software to evaluate the impact of freezing rate and cryoprotectants on performance and fertilization capacity of sperm stored in liquid nitrogen.[3] When this technique is perfected it will enable the long term storage of sperm of elite broodstock. For artificial inseminations conducted in the laboratory, the capacity for short- and long-term storage of male gametes will be an important element in the creation of families, and for the development of gene banks. In order to create a successful long-term storage or cryopreservation protocol for a given species, the diluents composition, cryoprotectant agent, cryoprotectant concentration, sperm packaging unit (e.g. cryostraw), sperm:extender ratio, sperm:extender equilibrium time, cooling rate, thawing rate, and storage vessel must be investigated. Adding to the complexity is the fact that all of these factors may interact with one another. CGP research is ongoing to improve current Atlantic cod cryopreservation procedures. Once protocols have been optimized we will then quantify how the biology/quality of the sperm is influencing post-thaw motility, morphology, and fertilization success. This research will contribute to the development of cryopreservation techniques for Atlantic cod, as well as for other gadoids. These studies will also be the first reported investigations in the fish cryopreservation literature that integrate sperm motility/velocity, morphology, ionic composition, and fertilization success.

## Genomics

The genomics component of the CGP has focused on the generation and use of molecular tools in Atlantic cod for use in the assessment of economically relevant traits in aquaculture, to quantify the diversity and health of wild populations and to develop biomarkers allowing the environmental impacts of current commercial seafood production to be determined (Figure 3). This has led to the production of ~158,000 expressed sequence tags (ESTs), all of which have been deposited in public databases and are thus freely available to the national and international research community. Several thousand genetic markers have also been identified, and these are being used to create a linkage map for Atlantic cod and to assess the genetic diversity of wild populations. The project is also currently testing a 20,000 element (20K) oligonucleotide

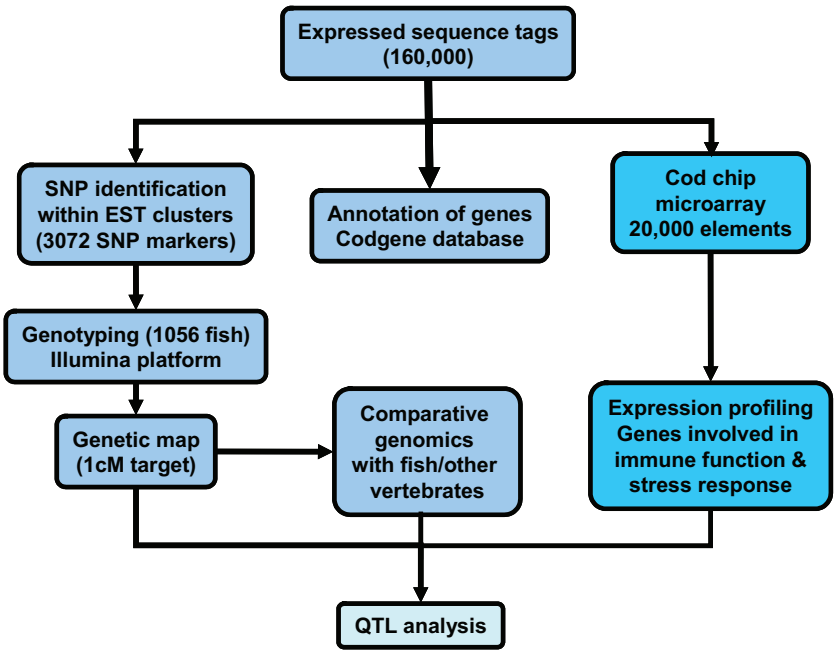


**Figure 3**
**CGP genomics tools development and applications.**

microarray for use in gene expression analyses. These tools are being applied to analyze traits of importance within the aquaculture industry, such as growth, and resistance to stress and disease, and tolerance to temperature changes.

The CGP was designed as an integrated selective breeding/genomics project, with samples for genomics research being taken from fish enrolled in the selective breeding programs to ensure that markers identified would be relevant to these programs. The development of single nucleotide polymorphisms (SNPs) through research efforts of the CGP has been extremely successful, with 1698 polymorphic SNPs and 155 microsatellite markers[4] identified from the CGP sequences. These are among the markers which are being used in the development of a 5.7 cM genetic map for cod. The extensive set of CGP ESTs were generated from libraries produced either from fish used as parents in the breeding programs or from F1 progeny within the breeding programs. These ESTs were aligned to form contiguous sequences (contigs), from which SNPs predicted to be highly variable within the breeding programs were identified. Two high-throughput genotyping panels (each comprising 1536 SNPs) were developed using Illumina GoldenGate technology. These panels were tested against fish from populations used as broodstock from years 1, 2 and 3 of the CGP, but also against additional Canadian and Northern European populations, and the SNPs were shown to be highly variable. These markers are currently being used to analyze quantitative trait loci (QTL), which will be used to accelerate selective breeding in Atlantic cod aquaculture.

We are also designing tools to assist the Canadian aquaculture industry with their fish breeding initiatives. Programs in family-based selective breeding manage their fish families using two approaches. In the first, fish families are reared in separate tanks (one per family) until they reach a suitable size for PIT tagging. This process involves inserting a transponder chip into the abdomen of each fish, allowing identification with a hand-held scanner. The second approach allows families to be pooled at a smaller size, and uses genotyping to assign fish to families at a later stage. Genotyping is commonly performed using a panel of five or more microsatellites. Unfortunately, microsatellite genotyping is relatively labour intensive, slow, and can be error prone due to polymerase stutter, incorrect allele calling and other issues. We are using our SNP collections to design panels for use in family identification, as SNPs are more easily incorporated into automated pipelines. However, as SNPs are almost invariably biallelic, it has been necessary to include more SNPs in a panel (> 30). We are genotyping a panel of 30-96 SNPs with the aim of identifying a minimal set of SNPs suitable for determining family ID. This panel will also be suitable for assessing relatedness within family breeding programs, to avoid crossing closely related individuals.

The CGP is using a functional genomics approach to identify and study the Atlantic cod genes involved in defense responses. This approach includes the development of several targeted cDNA libraries (e.g. suppression subtractive hybridization libraries) enriched for gene sequences that are involved in biological processes of interest to aquaculture research (e.g. responses to bacteria, viruses, or heat stress). The CGP has generated over 46,000 ESTs (project total ~ 158,000) from cDNA libraries that were designed for immune-relevant gene discovery.[5,6] The CGP EST set was used to develop the 20K cod microarray that will be an extremely useful tool for understanding the genes and molecular pathways involved in cod responses to pathogens and environmental stress. In addition, the CGP has developed quantitative reverse transcription – polymerase chain reaction (QPCR) assays for conducting detailed gene expression studies for several defense-relevant genes. These QPCR studies assess biological variability of gene expression

responses, allowing us to identify individuals with different levels of response (e.g. anti-bacterial gene expression response) to a given stimulus (e.g. bacterial antigens).[6] For many genes involved in stress and immune responses, the CGP is identifying SNPs (molecular markers) that may be suitable targets for selecting cod with superior traits (e.g. natural resistance to bacterial or viral infection).

The CGP has dramatically improved the availability of molecular tools for cod research. The resources being developed by the CGP will be invaluable for analyzing quantitative trait loci (QTL), identifying genes of potential importance in Canadian cod aquaculture and enabling marker assisted selection (MAS) within cod breeding programs. These markers will also be useful in management of wild stocks and fisheries, for example, in analyzing cod populations to compare the genetic variability within and between different designated fishing zones. We must continue to build on this solid foundation of cod genomics knowledge and expertise to ensure that the CGP-built resources are maximally utilized for improving the cod aquaculture industry in Canada.

### Genetic Improvement

The development of superior broodstock combines management and rearing of fish along with accurate estimates of the genetic merit of superior fish. The genetic improvement components of the project were two-fold: a) to develop statistical genomics tools to produce Estimated Breeding Values (EBVs), and b) to develop associations between molecular genetic markers and phenotypes of interest (QTL detection). Statistical genomics tools were developed to assess live fish growth traits and carcass characteristic traits. When computed with a technique know as Best Linear Unbiased Prediction (BLUP), EBVs account for all known ancestor information and the performance of relatives, offspring and descendents to determine the genetic merit of individuals and their suitability for use as broodstock. As part of this process, the heritability of each trait analysed was computed to determine the degree of inheritance of the characteristic and the resulting potential for genetic improvement.

Along with these conventional selection tools, marker assisted selection (MAS) tools were also developed. Fish were genotyped for a series of 1,100 single nucleotide polymorphisms (SNPs) and those genotypes were studied using similar statistical genomics tools developed to assess the association between the individual SNP marker alleles and superiority of phenotype. Developed in this way, the markers provide a "handle" to identify the fish with the best combination of genes to produce superior broodstock. This technique uses existing variation within the fish to identify superior individuals. The culmination of the genetic improvement component will be the identification of the optimal combination of EBVs and SNP genotypes to provide a marker assisted selection program for creating superior broodstock for many future year-class generations.

### Cod Stress Physiology

Fish in the wild generally have the ability to select environments that are favourable for maintaining good health. Features of the environment that may be selected for include water conditions (temperature, dissolved oxygen levels, current velocity, etc.) as well as social conditions (e.g. fish density). When maintained in net pens fish are sometimes unable to find/select environments that promote rapid growth, good health, and overall well-being.

This limited selection of environments means that fish in net pens can be exposed to stressful conditions (environmental and social) which they would nor-

mally avoid in the wild. Exposure of fish to stressors can cause physiological changes at multiple levels of animal organization; these alterations are collectively known as the stress response. The initial response to the perception of a stress is characterized by a neuroendocrine response, which includes the release of stress hormones such as cortisol.[7] In later stages the stress response can manifest itself in reduced growth, increased incidence of disease and, in severe cases, morbidity. Furthermore, high densities of fish within the confined space of the net pen can increase the likelihood of exposure to pathogens, which can also increase risk of disease.

CGP research activities include: a) studying the stress response of cod; b) developing biomarkers to accurately determine environmental conditions that are stressful for cod; c) assessing variability in the stress response between families, and its association with production relevant traits; and d) conducting novel experiments on the effects of stress on the cod's immune system. We have used classical physiological and molecular techniques in our ongoing studies of the stress response, studies of physiological performance,[8] as well as studies that examine the effects of acute and chronic thermal challenges on the immune response.[9] We have also conducted large-scale gene discovery and expression studies to understand which genes are involved in the physiological and cellular aspects of stress caused by factors such as acute increases in temperature.[10] Considerable variation among cod families with respect to their tolerance to elevated temperatures and stress has been identified which suggests that there is good potential to selectively breed Atlantic cod for optimal performance in net pens.

With increased focus on the culture of Atlantic cod it has become evident that there are several diseases that may severely limit the success of this industry. CGP research has focused on three pathogens: a virus (Atlantic cod nervous necrosis virus; nodavirus), a bacterium (atypical *Aeromonas salmonicida*) and a microsporidian parasite (*Loma morhua*). These pathogens have been responsible for disease outbreaks in farmed cod populations.[11-13] We are identifying key genes and molecular pathways involved in the innate and adaptive immune responses of Atlantic cod to these pathogens.[5] The present focus of our research in this area is to understand the role that environmental stressors play in the switch from the carrier to disease state, and to utilize the newly developed 20K cod microarray to study the iterative effects of environmental conditions, stress, and pathogen exposure on global gene expression responses in immune relevant tissues. As with the stress response, a considerable amount of variation among cod families in their resistance to disease has been identified, suggesting that selective breeding may result in more disease resistant Atlantic cod for the industry.

## GE$^3$LS

The CGP includes an element of research on Ethical, Economic, Environmental, Legal and Social issues associated with the science of Genomics (GE$^3$LS). The GE$^3$LS research team is developing solution-oriented legal and policy options regarding: legal ownership of commercially valuable research results, the status of elite cod broodstock under Canadian environmental law and Canada's international obligations, and ethical and legal options regarding benefit sharing and improved methods of consultation with the affected publics.[14,15] A recent book generated through the efforts of the research team, *Aquaculture, Innovation and Social Transformation*,[16] presents and interprets Canadian and international perspectives on the debate over the future of aquaculture in Canada. The following quotation regarding this book is from the publisher's online description.[17] "Orig-

inal chapters examine: animal welfare; knowledge management and intellectual property; environmental sustainability; local, traditional, and aboriginal knowledge; consumers and integrated coastal zone management. Authors of principal chapters are drawn from Canadian and European universities, while commentators are drawn from Canadian government and private sectors. This structure results in a deliberately engineered collision of diverse habits of thought and dissimilar bases of knowledge. In that collision the problems, options, and possible future of aquaculture are both explicitly argued, and shown in the interaction between authors and perspectives. Of particular note is the inclusion of perspectives written by First Nations members, and an epilogue from the comparative perspective of the US experience."

In addition, the CGP GE$^3$LS team has sought opportunities for collaboration with the Genome Canada/Genome BC consortium for Genomic Research on All Salmonids Project (cGRASP) GE$^3$LS team, which is examining various topics related to salmonids. The teams recognized that each GE$^3$LS group is concerned with social issues raised by genomic research in fish that have special, iconic social significance. Further, they recognized that the general public is increasingly interested in the interaction between capture and culture fisheries, and what genomics and applied biotechnology can do to help integrate those fisheries to serve diverse goals of conservation of capture stocks and support of coastal communities. Collaboration between these teams has enhanced east-west communication, and served as a nucleus to draw other Canadian and international researchers' attention to their research questions.


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# Application of Salmonid Genomic Research in a Mid-Sized Commercial Broodstock Facility: The Icy Waters Arctic Charr Experience

Colin McGowan

**C. McGowan, J. Lucas, E. Keddie, J. Rose, E. Davidson, N. Quinn and W.S. Davidson**

Icy Waters Ltd. is a privately-owned Canadian company and a world leader in Arctic charr (*Salvelinus alpinus*) aquaculture. Located in Whitehorse, Yukon Territory, it is a fully-integrated operation that includes a DFO-certified broodstock facility, hatchery, tank farm and CIFA-approved processing plant. Icy Waters also sells *ova* to aquaculture operations throughout the world. In 2001, Icy Waters embarked on a six-year collaboration with Simon Fraser University (SFU) to incorporate new technologies from the fields of molecular genetics and genomics into their breeding strategy. Genetic markers have been used to determine the genetic relationship between two purebred strains (Nauyuk Lake and Tree River), avoid inbreeding, and resolve pedigrees for the estimation of genetic parameters such as heritability. Markers that identify sex are being used to generate all-female lines of broodstock and production fish. A genome-mapping project has identified QTLs for early growth rate that have been used in a marker-assisted selection program. More recently, the company's interests have turned towards the discovery of genetic markers that are associated with disease and stress resistance. We continue to collaborate with SFU and are involved in projects investigating gene expression at high water temperatures and the evolution of the salmonid sex chromosome.

## Introduction

### *Background—Icy Waters Ltd.*

Icy Waters Ltd. is a privately-owned Canadian company and a world leader in Arctic charr (*Salvelinus alpinus*) aquaculture. It is committed to the belief that Arctic charr are one of the finest freshwater finfish available in the marketplace. Located in Whitehorse, Yukon Territory, Icy Waters is a fully integrated operation that includes a DFO-certified broodstock facility, hatchery, tank farm and CIFA-approved processing plant.

**Yukon Gold™ Ova**

Annual production of flesh exceeds 120 metric tons and is sold to restaurants and retail establishments across North America. Icy Waters also sells ova to aquaculture operations throughout the world.

Icy Waters is committed to the objective of environmentally sustainable aquaculture. As a land-based facility, interactions with wild populations of fish, and the potential for the transfer of disease or genetic material between wild and cultured stocks are negligible. The gravity-fed water source and flow-through design eliminate the energy requirement of pumps. Drum filters, settling ponds and wetlands are used to remove particulate matter and effluent before returning the water to the natural wetlands, downstream of the facility.

Icy Waters has been growing Arctic charr for over 20 years. These fish are extremely fun to grow and breed—they are hardy, prefer high stocking densities (80 to 120 kg/m$^3$), have an attractive colourful skin, and accept pigments readily, producing a mild tasting orange-coloured flesh high in omega-3 fatty acids. Arctic charr are a relative newcomer to the aquaculture industry and still possess many of their wild attributes and genetic potential. Still, as with most commercially-grown finfish, their propagation is not without challenges—including maintaining and managing genetic diversity within the broodstock, improving growth rates, preventing early maturation in males, and improving tolerance of warm temperatures and resistance to disease. Many of these challenges are being met through selective breeding, where permanent improvements to our broodstock benefit production at our Whitehorse facility as well as at the facilities of our many ova customers worldwide, who grow Arctic charr from Icy Waters.

### Icy Waters Broodstock Program

Two purebred strains of Arctic charr are maintained at the Icy Waters White-

**Table 1**

**Genetic diversity (A) and observed heterozygosity ($H_0$) of Tree River (TR), Nauyuk Lake (NL) and their reciprocal hybrids.**

| Marker | | TRf x TRm (n = 250) | NLf x NLm (n = 210) | TRf x NLm (n = 186) | NLf x TRm (n = 203) |
|---|---|---|---|---|---|
| SalE38SFU | A | 6 | 4 | 8 | 8 |
| | $H_o$ | 0.77 | 0.63 | 0.98 | 0.95 |
| Sfo8Lav | A | 4 | 4 | 7 | 7 |
| | $H_o$ | 0.59 | 0.82 | 1 | 0.99 |
| Ssa85DU | A | 6 | 3 | 6 | 5 |
| | $H_o$ | 0.76 | 0.03 | 0.69 | 0.56 |
| Sfo23Lav | A | 4 | 5 | 7 | 9 |
| | $H_o$ | 0.68 | 0.64 | 0.99 | 0.99 |
| One8ASC | A | 6 | 6 | 7 | 7 |
| | $H_o$ | 0.26 | 0.8 | 0.94 | 0.96 |
| SalP61SFU | A | 9 | 7 | 9 | 9 |
| | $H_o$ | 0.61 | 0.79 | 0.79 | 0.82 |
| SalD39SFU | A | 10 | 5 | 11 | 9 |
| | $H_o$ | 0.89 | 0.49 | 0.97 | 0.79 |
| Sal5UG | A | 10 | 6 | 10 | 9 |
| | $H_o$ | 0.73 | 0.43 | 0.95 | 0.98 |
| A (total) | | 55 | 41 | 65 | 63 |
| A (mean) | | 6.9 | 5.1 | 8.1 | 7.9 |
| $H_o$ (mean) | | 0.66 | 0.58 | 0.91 | 0.88 |

horse facility. Both are from the Western Arctic of North America and came to Icy Waters through the Rockwood Aquaculture Research Center in Gunton, Manitoba, Canada. The Nauyuk Lake (68°20′N, 107°20′W) Arctic charr were collected from the wild in 1978 and the Tree River (67°40′N, 111°51′W) strain was collected in 1988.[1] Nauyuk Lake Arctic charr are commonly used in aquaculture, and represent the standard with regards to size, shape, colour and flesh quality for commercially-available Arctic charr in North America.[2] The Tree River strain is one of the fastest-growing and largest Arctic charr strains in the world,[3,4] but suffers from pale flesh colour, early maturation of males, and poor disease resistance compared to the Nauyuk Lake strain. A hybrid combination of these two strains is grown in flesh production facilities throughout the world and represents approximately 80% of the Arctic charr grown in North America.[5]

A line of hybrid broodstock is also maintained in Whitehorse. These fish are used predominantly for the production of backcross families used to map quantitative trait loci (QTL) of commercial interest in the genomes of Nauyuk Lake and Tree River Arctic charr. They are also used to generate an all-female, triploid backcross line that has performed well under commercial production.

There are two spawning seasons at Icy Waters. The spring spawn is undertaken with photo-manipulated broodstock and is used mainly for the commercial pro-

duction of triploid hybrid ova for sale to hatcheries and flesh production facilities throughout the world, or for our own hatchery and flesh production facility. In the fall, the natural spawning time for Arctic charr, in addition to our production broodstock, we maintain several select lines that form the foundation of our breeding program and where the majority of our genetic research is concentrated.

Arctic charr females require about five years before they are ready to start producing eggs. Once they have reached maturity, they can be reconditioned to produce eggs for several subsequent years. Consequently, breeding requires careful planning and patience, but the benefits of good breeding can be exploited over many years. Some of our select lines include Arctic charr that have been selected for growth using marker-assisted selection, all-female lines for the production of all-female production populations, and broodstock that have been selected for their tolerance of warm water temperatures. We are also planning to establish a line of Arctic charr with improved resistance to furunculosis (*Aeromonas salmonicidia*).

## Genomics at Icy Waters

Since its establishment in 1986, a strong broodstock development program has been a cornerstone of Icy Water's success. In 2001, Icy Waters embarked on a 6-year collaboration with Simon Fraser University to incorporate new technologies from the field of molecular genetics and genomics into their breeding strategy. At the time, the first linkage maps for Arctic charr were being completed at the University of Guelph,[6] giving the project the rudimentary information it needed to get started. Over the years, we have used this strategy to maintain and manage genetic diversity within our purebred lines, identify QTLs and perform marker-assisted selection (MAS) for growth rate, and create pedigrees and manage sex ratios. Genomics continues to play a role in projects aimed at improving upper temperature tolerance and resistance to furunculosis. The following is an overview of the genetic research program currently underway at the Icy Waters Whitehorse facility.

**Figure 1**
**Small section of a relatedness matrix.**

**Individual Tag Numbers**

**Unrelated Individuals**
r < 0.0405 i% confidence)
r < -0.158 I% confidence)

| | 490155 | 491118 | 492241 | 492311 | 492455 | 492562 | 492563 | 494527 | 494824 | 495037 | 495757 | 497149 | 497152 | 497154 | 497164 | 497165 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 490155 | * | 0.313165 | 0.393046 | 0.118493 | 0.190328 | -0.368412 | -0.093443 | 0.074539 | -0.052752 | 0.091185 | -0.024379 | -0.235387 | 0.3577 | 0.144129 | 0.579288 | -0.266453 |
| 491118 | 0.313165 | * | -0.264678 | -0.02769 | 0.352045 | -0.244994 | -0.063376 | -0.064529 | -0.019964 | 0.474982 | -0.000781 | -0.291411 | 0.760362 | -0.425753 | -0.165737 | -0.410343 |
| 492241 | 0.393046 | -0.264678 | * | -0.268935 | -0.272053 | -0.376514 | -0.243197 | -0.025557 | -0.134099 | -0.300177 | -0.110433 | -0.114507 | -0.307923 | 0.763044 | 0.352403 | 0.062449 |
| 492311 | 0.118493 | -0.02769 | -0.268935 | * | 0.068013 | 0.466988 | 0.19922 | 0.323425 | 0.049012 | 0.168038 | 0.257944 | -0.11836 | -0.039765 | -0.464191 | -0.141268 | -0.313457 |
| 492455 | 0.190328 | 0.352045 | -0.272053 | 0.068013 | * | -0.234982 | 0.571581 | 0.608504 | 0.326475 | 0.062343 | -0.090907 | 0.348125 | 0.670009 | -0.519491 | -0.104855 | -0.516008 |
| 492562 | -0.368412 | -0.244994 | -0.376514 | 0.466988 | -0.234982 | * | 0.02272 | 0.066578 | 0.149762 | -0.10003 | 0.37982 | 0.376852 | -0.29517 | -0.436873 | -0.488789 | 0.150616 |
| 492563 | -0.093443 | -0.063376 | -0.243197 | 0.19922 | 0.571581 | 0.02272 | * | 0.173759 | -0.023323 | 0.416803 | -0.258363 | -0.009315 | 0.354633 | -0.389902 | -0.279412 | -0.376289 |
| 494527 | 0.074539 | -0.064529 | -0.025557 | 0.323425 | 0.608504 | 0.066578 | 0.173759 | * | 0.599349 | -0.04542 | 0.414318 | 0.607872 | 0.071795 | -0.24212 | -0.002184 | -0.088956 |
| 494824 | -0.052752 | -0.019964 | -0.134099 | 0.049012 | 0.326475 | 0.149762 | -0.023323 | 0.599349 | * | -0.37663 | 0.776354 | 0.55027 | 0.139714 | -0.213297 | -0.38419 | 0.103889 |
| 495037 | 0.091185 | 0.474982 | -0.300177 | 0.168038 | 0.062343 | -0.10003 | 0.416803 | -0.04542 | -0.37663 | * | -0.341914 | -0.347869 | 0.390749 | -0.498178 | -0.179506 | -0.478121 |
| 495757 | -0.024379 | -0.000781 | -0.110433 | 0.257944 | -0.090907 | 0.37982 | -0.258363 | 0.414318 | 0.776354 | -0.341914 | * | 0.344617 | -0.009697 | -0.189564 | -0.34342 | 0.121156 |
| 497149 | -0.235387 | -0.291411 | -0.114507 | -0.11836 | 0.348125 | 0.376852 | -0.009315 | 0.607872 | 0.55027 | -0.347869 | 0.344617 | * | -0.182801 | -0.193655 | -0.125024 | 0.412326 |
| 497152 | 0.3577 | 0.760362 | -0.307923 | -0.039765 | 0.670009 | -0.29517 | 0.354633 | 0.071795 | 0.139714 | 0.390749 | -0.009697 | -0.182801 | * | -0.487757 | -0.204725 | -0.469605 |
| 497154 | 0.144129 | -0.425753 | 0.763044 | -0.464191 | -0.519491 | -0.436873 | -0.389902 | -0.24212 | -0.213297 | -0.498178 | -0.189564 | -0.193655 | -0.487757 | * | 0.393863 | 0.218448 |
| 497164 | 0.579288 | -0.165737 | 0.352403 | -0.141268 | -0.104855 | -0.488789 | -0.279412 | -0.002184 | -0.38419 | -0.179506 | -0.34342 | -0.125024 | -0.204725 | 0.393863 | * | -0.045522 |
| 497165 | -0.266453 | -0.410343 | 0.062449 | -0.313457 | -0.516008 | 0.150616 | -0.376289 | -0.088956 | 0.103889 | -0.478121 | 0.121156 | 0.412326 | -0.469605 | 0.218448 | -0.045522 | * |

**Table 2**

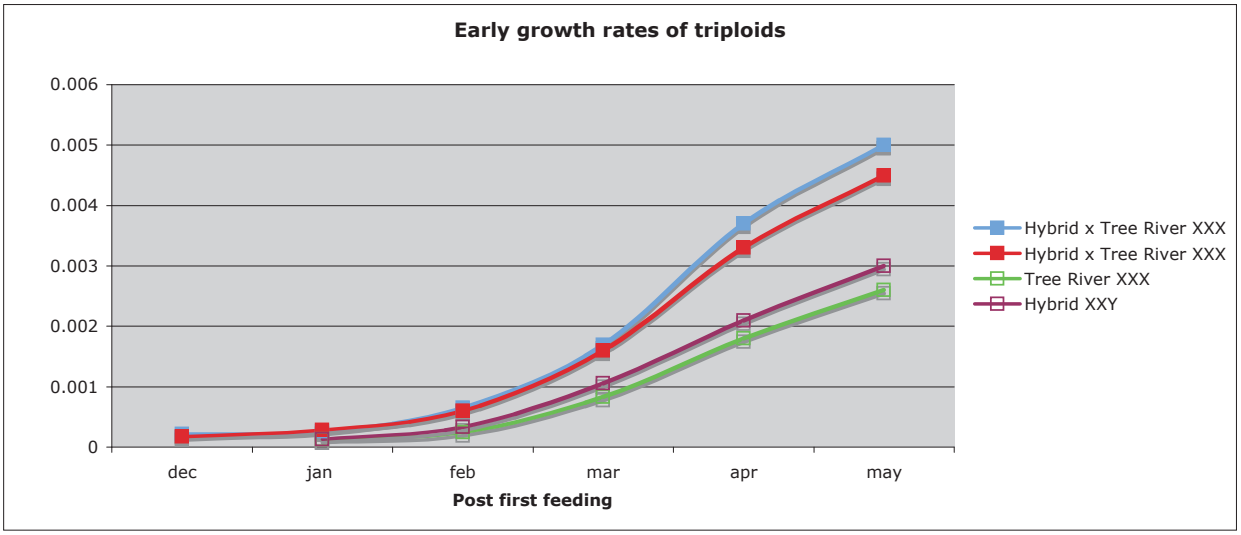
**Mean body weight (BW), fork length (FL) and condition factor (K) for twelve different cross combinations generated in the fall of 2001 (standard deviation in brackets). Samples were taken at 850 degree-days post first-feeding.**

| Cross | mean BW (g) | mean TL (cm) | mean K |
|---|---|---|---|
| NLf x NLm | 1.02 (0.16) | 5.1 (0.27) | 0.76 (0.06) |
| (NLf x TRm)f x NLm | 1.09 (0.22) | 5.2 (0.34) | 0.76 (0.08) |
| (TRf x NLm)f x NLm | 1.11 (0.26) | 5.3 (0.41) | 0.73 (0.07) |
| NLf x (NLf x TRm)m | 0.98 (0.23) | 5.1 (0.40) | 0.73 (0.06) |
| NLf x (TRf x NLm)m | 1.20 (0.32) | 5.5 (0.47) | 0.71 (0.06) |
| NLf x TRm | 1.35 (0.28) | 5.8 (0.38) | 0.69 (0.04) |
| TRf x NLm | 1.34 (0.30) | 5.6 (0.44) | 0.76 (0.07) |
| TRf x (TRf x NLm)m | 1.29 (0.29) | 5.6 (0.41) | 0.74 (0.09) |
| TRf x (NLf x TRm)m | 1.28 (0.36) | 5.6 (0.50) | 0.70 (0.06) |
| (NLf x TRm)f x TRm | 1.50 (0.40) | 5.9 (0.50) | 0.70 (0.05) |
| (TRf x NLm)f x TRm | 1.53 (0.35) | 6.0 (0.42) | 0.70 (0.06) |
| TRf x TRm | 1.75 (0.50) | 6.1 (0.53) | 0.74 (0.05) |

***Management of genetic variation***

Some of the earliest goals for our genetics program were to establish baseline genetic information for future reference, prevent a loss of genetic variation, prevent inbreeding and promote heterozygosity within our lines. In the spring of 2001, our 1996 broodstock year-class was screened with eight different microsatellite markers (Table 1). This survey was carried out to obtain baseline information about the level of genetic variation present within the Icy Waters

**Figure 2**
**Growth rates of an all-female triploid backcross compared with the standard hybrid triploid and the all-female Tree River triploid.**



broodstock lines, to estimate the relatedness of breeding pairs, and to provide genetic fingerprints for pedigree analysis.

Allele, or genetic, diversity varied between lines, with the Tree River line being more diverse than the Nauyuk Lake line. The Tree River line has been domesticated for fewer generations than the Nauyuk Lake line,[1] which may be reflected in this difference. The two purebred lines were also found to be genetically distinct ($F_{st}$ = 0.351). Consequently, hybrid broodstock and production fish have a relatively high observed heterozygosity and the overall genetic diversity is strong. This might account, in part, for the strong performance of hybrid fish under commercial conditions.

In an effort to improve heterozygosity within the purebred lines, the genetic data were used to estimate the genetic relationship between breeding pairs. The computer program Relatedness[7] was used to estimate the genetic relationship between individual pairs within a population so that crosses between closely-related individuals could be avoided (Figure 1). The results of these crosses have performed well under commercial conditions. However, the success of this approach to improve the genetic heterozygosity of a line will be measured this fall when the resulting year-classes of broodstock are tagged and sampled.

**Table 3**

**Regression of mean body weight with genotype for a population of backcross fish.**

**Hybrid Genotypes**

| Marker | Genotype Class (n) | | Respective means (SD) | | P |
|---|---|---|---|---|---|
| OmyRGT39 | 106/116 (48) | 106/118 (18) | 24.52 (10.48) | 30.19 (13.65) | 0.0635 |
| | 106/116 (48) | 106/110 (20) | 24.52 (10.48) | 15.12 (10.37) | 0.0008 |
| | 106/118 (18) | 106/110 (20) | 30.19 (13.65) | 15.12 (10.37) | 0.0006 |
| OmyRGT4 | 132/142 (22) | 142/140 (7) | 27.62 (14.56) | 37.80 (13.03) | 0.0515 |
| | 132/142 (22) | 140/150 (23) | 27.62 (14.56) | 15.03 (8.535) | 0.0015 |
| | 142/140 (7) | 140/150 (23) | 37.80 (13.03) | 15.03 (8.535) | 0.0003 |
| OMM1037 | 122/124n (30) | 122/128 (12) | 21.48 (10.76) | 19.76 (9.39) | 0.385 |
| | 122/124n (30) | 124t/128 (38) | 21.48 (10.76) | 23.54 (10.80) | 0.2075 |
| | 122/128 (12) | 124t/128 (38) | 19.76 (9.39) | 23.54 (10.80) | 0.1403 |

**Tree River Genotypes**

| Marker | Genotype Class (n) | | Respective means (SD) | | P |
|---|---|---|---|---|---|
| OmyRGT39 | 116/118 (50) | 118/110 (21) | 20.86 (10.18) | 20.20 (14.29) | 0.174 |
| | 116/118 (50) | 118/118 (16) | 20.86 (10.18) | 29.26 (16.69) | 0.0255 |
| | 118/110 (21) | 118/118 (16) | 20.20 (14.29) | 29.26 (16.69) | 0.0223 |
| OmyRGT4 | 132/140 (38) | 140/140 (21) | 19.54 (12.84) | 21.28 (10.24) | 0.1374 |
| OMM1037 | 122/122 (40) | 122/124 (19) | 24.24 (12.07) | 8.89 (4.4) | <0.0001 |
| | 122/122 (40) | 124/124 (25) | 24.24 (12.07) | 31.49 (15.54) | 0.0287 |
| | 122/124 (19) | 124/124 (25) | 8.89 (4.4) | 31.49 (15.54) | <0.0001 |

***Improvement of early growth rates***

In the hatchery, the first year of life and growth can be a critical stage for a young fish. Small fish are susceptible to outbreaks of disease and tanks often require higher maintenance. Growing the fish quickly through the first year of life can lead to significant savings for a hatchery and can prevent significant losses. The discovery of genetic markers that are associated with quantitative trait loci (QTLs) for early growth traits can help to guide a breeder when selecting future broodstock. Genetic markers can be used in marker-assisted selection (MAS), introgression of genes from one strain into the genetic background of another strain, or to decrease the amount of genetic variability in a particular region of the genome, while maintaining overall variability. The goal of this project was to identify genetic markers associated with early growth traits in the Nauyuk Lake and Tree River strains and use them to perform MAS.

The 2001 year-class was also used to evaluate the early growth and performance of various backcross combinations and for genetic linkage analysis (Table 2). The crosses were followed over a period of two years with tissue samples taken at six months and 24 months post first-feeding. Early growth rates of hybrids backcrossed with Tree River were strong. Although they exhibited some of the less favorable characteristics of the Tree River strain, such as early maturation of males, they performed particularly well as all-female triploids (Figure 2). This specific configuration is currently being evaluated under commercial production conditions.

Markers used in the initial studies on variability were now used to perform pedigree analysis on several backcross lines to identify full-sibling families. Eight families were selected for the construction of genetic linkage maps for both the Tree River and Nauyuk Lake strains. Our approach was to generate linkage maps for single families using amplified fragment length polymorphisms (AFLPs) and microsatellite markers. Microsatellite markers were used to create continuity between the Icy Waters genetic maps and other salmonid genetic linkage maps. AFLP markers, though less informative from a comparative genomics outlook, gave density to the maps over a short period of time. As the maps were being assembled and analyzed, several QTLs for early growth rate were identified. Microsatellite markers demonstrating a significant association with growth rate were chosen for marker-assisted selection trials (Table 3). The aim of these experiments was to determine how effective we could be at altering allele frequencies in a population and, secondly, to determine if early growth rates could be improved with this approach.

**Icy Waters aquaculture facility in Whitehorse, Yukon.**

Icy Waters conducted two marker-assisted selection experiments. The first trial took place in the fall of 2005. At the time, we had only two microsatellite markers in hand that were associated with growth and were ready to test (*Omy*RGT39 and BHMS121). Our plan was to first see if we could effectively select individuals to breed that that would shift the genetic composition of the resulting population in a preferred direction. We would then monitor the growth of the different populations to see if a particular change in allele frequency resulted in the desired effect. Two lines of hybrid fish—a fast-growing line and a slow-growing line—were generated using purebred parents selected according to genotype. An unselected control group was also included in the trial. Once the resulting offspring had grown a bit, a sample from each of the three populations was taken and screened with the genetic markers to see if any changes in allele frequency had been realized.

The results of this experiment were mixed. Although some changes in allele frequency were achieved, they were not as drastic as had been hoped for and were not always in the right direction. For example, one goal with the fast-growing group was to increase the frequency of the 118 allele at the *Omy*RGT39 marker. In the resulting 'fast' population, this allele was no longer present and had been eliminated by random drift. Early growth results over the first 4 months were promising; however, as time went on, all groups were observed to grow at approximately the same rate.[8] It is possible that as fish continued to grow, density dependant factors had a predominating effect on growth rates.

**"The true value of marker-assisted selection is more likely to be realized for traits that are difficult to measure such as disease resistance or sex at an early age."**

The second marker-assisted selection trial was initiated in the fall of 2006 and is still ongoing. It is much more sophisticated than the first trial. In this case, three markers associated with growth were selected (*Omy*RGT4, *Omy*RGT39 and OMM1037). These markers were more heavily scrutinized than those used in the earlier project and are expected to have a greater effect. To simplify the selection process in the hatchery, information about the markers was combined with the relatedness matrix to create a breeding index for each potential pair. First, the entire broodstock was genotyped at four random markers to create a relatedness matrix. Then each individual was genotyped at the selected markers and ranked according to their genotype. Finally, the rankings were combined with the relatedness matrix to produce a breeding value for each potential pair of individuals.

Four groups were generated for this project, a select and control group for each of two backcross lines, a hybrid female backcrossed with a Tree River male, and a Tree River female backcrossed with a hybrid male. Again, growth rates over the first six months were promising, but have been hampered by variation in fish densities in the tanks.

These trials suggest that marker-assisted selection may not be the most effective approach to improving growth rates in Arctic charr. It is likely that many genes of minor effect rather than a few genes of major effect govern this trait. Also, our genetic maps have indicated that QTLs for growth are not the same at six months and 24 months post first-feeding. Consequently, a more traditional approach to selection for growth rate may be more effective. The true value of marker-assisted selection is more likely to be realized for traits that are difficult to measure such as disease resistance or sex at an early age.

#### *Production of all-female populations*

One of the current constraints on the developing Arctic charr industry is the early maturation of males prior to reaching market size. The transfer of energy stores from muscle to the developing gonads results in a loss of growth and poor flesh quality. This problem, which is common among salmonid species, may be

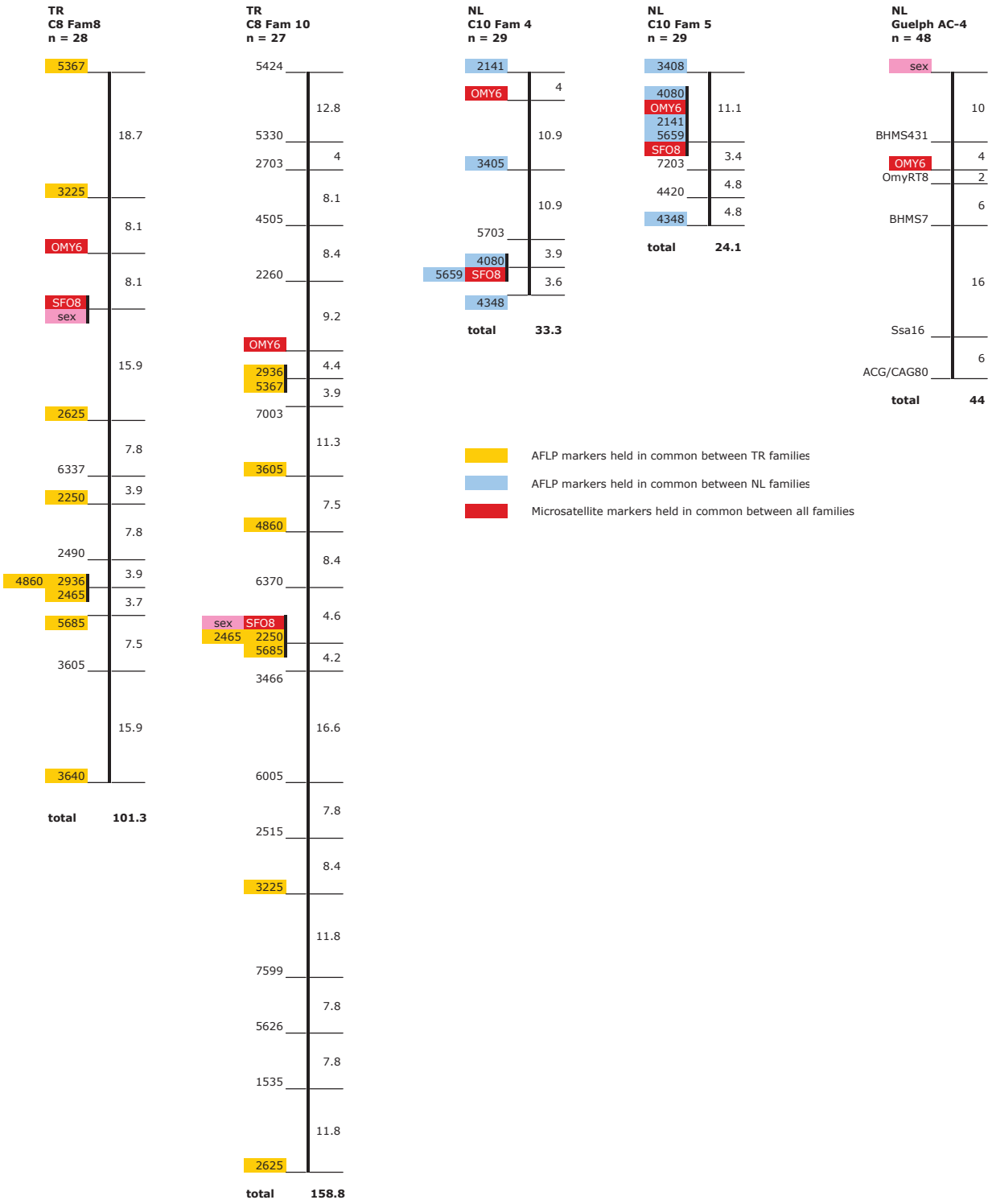
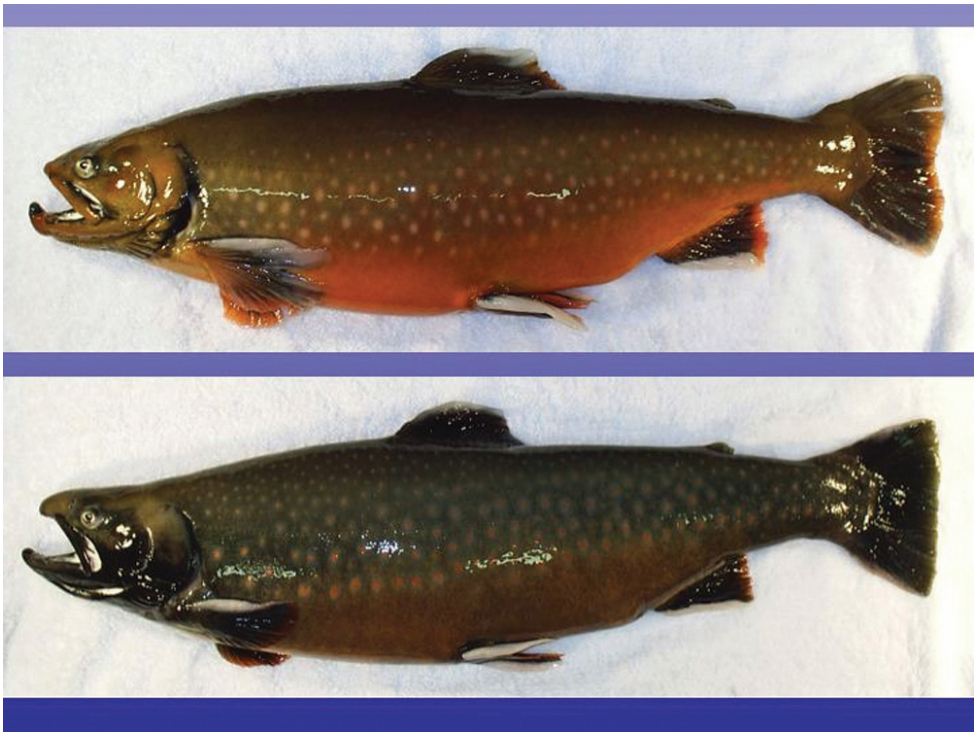



**Figure 3**
**Linkage maps of the Tree River and Nauyuk Lake sex chromosome.**

overcome by generating 'all-female' populations for production through the process of indirect feminization. Typically, broodstock fish are treated with testosterone prior to gonadal differentiation. This results in the functional masculinization of the females, whereupon the genotypic females (XX) develop masculine gonads and are capable of producing milt. Upon maturity, the milt can be removed from masculinized females and used to fertilize regular ova, resulting in an all-female production population.

In Arctic charr, masculinization of females may be achieved by feeding juvenile fish testosterone at first feeding for a period of approximately 600 degree-days after egg sac absorption.[9] The resultant population, a mix of genotypic males (XY) and masculinized females (XX), must be grown to maturity before secondary sexual characteristics develop and the two groups can be separated. At this stage, masculinized females are identified typically by the development of male secondary sexual characteristics and, after dissection, the presence of deformed gonads that prevent the normal venting of milt. The dissected gonads are then crushed and used to fertilize ova for the production of all-female populations.

This process can be costly and is not always certain. Since masculinized females cannot be identified until sexual maturation, the entire population of testosterone treated fish, both true males and masculinized females, must be raised to adulthood. In Arctic charr, this can be a period of up to five years, during which time the superfluous genotypic males must be fed and maintained and will generate unnecessary metabolic waste. In addition, presumed all-female populations produced at Icy Waters tend to consist of 20% males that mature early. In other words, approximately one in five adult fish identified as a masculinized female are actually normal, genotypic males with deformed gonads. A genetic marker that can identify the sex of a fish at an early age would help solve these problems and enable Icy Waters to offer its ova customers a guaranteed all-female population for commercial production.



**Adult male charr: Nauyuk Lake (above) and Tree River (below) strains.**

During the initial genetic analysis of the 1996 broodstock, it was observed that a microsatellite marker (*Sfo*8Lav),[10] initially chosen for pedigree analysis, exhibited a high degree of correlation with sex. Further study demonstrated that the marker could be used to identify sex in 100% of the Tree River adults tested (n = 250) and 95% of the Nauyuk Lake adults tested (n = 210). In addition, the marker was able to predict sex 100% of the time in hybrid adults when a Tree River fish is the male parent (n = 203). Since this analysis, several 'false positives' have been identified, indicating that linkage between this marker and the sex-determining factor is not absolute. However, in practical terms, linkage is tight enough for us to accept this marker as a strong indicator of sex in our populations of Arctic charr when the male line is descended from the Tree River strain. Our goal now is to use this genetic marker to identify the sex of juvenile fish in a population of testosterone-treated Arctic charr broodstock. Once identified, the unwanted males will be removed from the broodstock. The marker can also be used to screen and confirm that an all-female population is indeed all female. The effect of sorting and removing genotypic males from these populations will be a lasting one. That is, the guaranteed, all-female broodstock will be used to make both regular and masculinized females that will be crossed with each other to generate our future all-female production populations, without the risk of producing unwanted males.

In addition to the practical implications of this work, interesting scientific observations have also resulted. Linkage maps for areas of the genome that surround the Sfo8 locus were constructed for both the Tree River and Nauyuk Lake sex chromosome and compared with the genetic linkage map for the Nauyuk Lake sex chromosome generated at the University of Guelph[1] (Figure 2). Although several AFLP markers were conserved between families within each line, the order of these markers was not conserved. This ties in with observations of instability in chromosomal regions surrounding the sex determining factor in other salmonids.[11] Also, while all the Nauyuk Lake linkage groups were comparable in size, the Tree River groups were much longer. This apparent difference in size could be the result of differences in the rates of recombination between the two strains, or it could reflect a physical difference between the Tree River and the Nauyuk Lake Y-chromosome. We hope to pursue this question in the future using fluorescent in-situ hybridization (FISH).

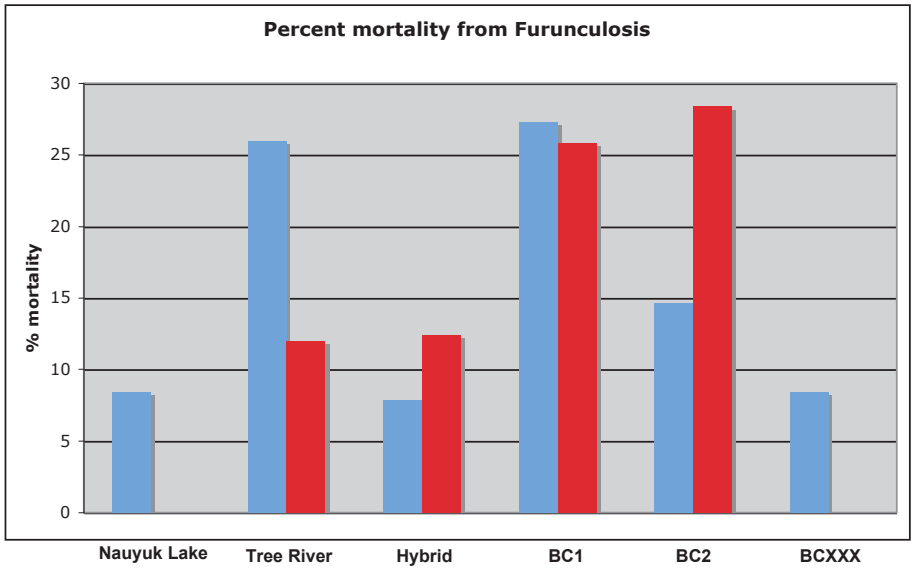



**Figure 4**
**Mortality due to furunculosis during the summer of 2008. Blue and red bars represent duplicate trials.**

### *Upper temperature tolerance*

The natural range of Arctic charr is the most northerly of all the salmonid species. Consequently, intensive culture is limited to regions where water temperatures are not expected to exceed 15°C on a regular basis. Even in water-recirculation facilities, additional energy is required to maintain op-

timal temperatures, making the culture of this species less attractive to growers in more southerly latitudes. At our facility in the Yukon, summertime mortality resulting from heat stress averages 8 metric tons per year. This represents a financial loss of over $80,000 per summer. During a heat wave in 2004, water temperatures regularly exceeded 20°C and mortality between May and September was over 24 metric tons. When extra labour, oxygen and reduced growth were accounted for, the company lost over $400,000 in one summer. Warm water also places our broodstock at risk and affects the quality of the eggs. Therefore, understanding how fish deal with warm water and how to limit or prevent summertime mortality is a critical issue for our company.

Our current collaboration with Simon Fraser University uses genomics to help develop greater temperature tolerance in our broodstock. Here, the approach is to first identify genes that help protect an individual during the periods of stress that can result from warm water temperatures. A 32K microarray developed for salmonids by cGRASP[12] has been used to screen the expression patterns of fish exposed to both acute and chronic heat stress. So far, 84 genes that are only expressed in fish with high upper temperature tolerance have been identified. Heat shock proteins and various globin genes figure prominently on this list. Our goal is to use comparative genomics and the Atlantic salmon genetic map to identify genetic markers for regions of the Arctic charr genome that might be responsible for protection during heat stress. These markers will be used to screen families of Arctic charr and search for allelic differences between heat sensitive and heat tolerant fish. If all goes well, strong candidate markers will be used in future rounds of marker-assisted selection.

By understanding the mechanism by which Arctic charr or other salmonids can endure warm water temperatures and by identifying regions of the genome responsible for upper temperature tolerance, we hope to breed a strain of Arctic charr that is more resistant to temperature stress. This will benefit Icy Waters directly by decreasing the rate of mortality during the summer months and limiting the associated financial losses. It will also broaden our potential market for ova sales by allowing producers to grow Arctic charr in regions that were previously considered unsuitable.

#### *Future directions—disease resistance*

As a species, Arctic charr are a hardy fish with good overall disease resistance. However, furunculosis (*Aeromonas salmonicida*) is a widespread problem and can result in significant losses to a fish farm. Although our vaccination program is highly effective, it is costly and we are constantly looking for ways to improve the odds. Recent studies in Atlantic salmon (*Salmo salar*) have indicated that disease resistance is heritable and can be improved upon with selection.[13]

One of our future research goals is to create a line of Arctic charr with improved resistance to furunculosis, or disease in general. Preliminary research at our facility during the summer of 2008 identified between-strain differences in resistance to furunculosis (Figure 4). The Nauyuk Lake strain and the hybrids tend to have the best resistance, whereas the Tree River strain and backcrosses to Tree River, have the worst. Interestingly, the triploid all-female backcross, which was also a fast grower, performed much better than their normal, mixed sex, diploid counterparts. Vaccination reduces mortality to close to zero in all strains, with the best results in hybrids.

The current objective of this project is to estimate the heritability of resistance to furunculosis for our Arctic charr populations. This fall we will be crossing hy-

brids to generate several groups of half-sibling families that will be grown together in a single tank, or common garden. During a summer of exposure to the disease without vaccination, the groups will be sampled and separated into families by using genetic markers to perform pedigree analysis. If estimates of heritability are favorable, we can screen the families for QTLs that can be incorporated into our marker-assisted selection program. One of the problems with selecting for disease resistance in fish is that the resistant individuals, who have been exposed to a pathogen, cannot be introduced to the broodstock. Genetic markers offer the means by which this information can be brought back to the hatchery.


### Acknowledgements

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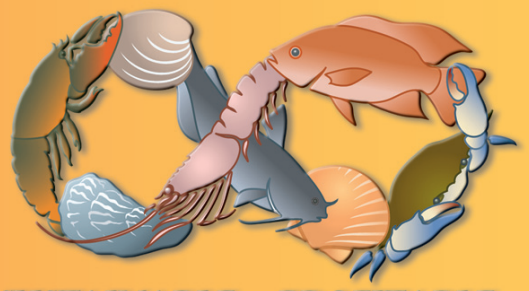

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# The *Myt*-OME Project: Development of a Health Assessment Tool for Marine Mussels

Helen Gurney-Smith

**Helen Gurney-Smith and Stewart Johnson**

The British Columbia coastline is under increasing pressure from competing coastal zone utilization (e.g., urbanization, recreation and aquaculture) and potential climate change impacts, highlighting the need for effective diagnostic tools of coastal ecosystem health and function. For cultured and wild shellfish, a variety of environmental, biological and human factors have been identified that could have significant effects on these populations. Detailed studies on the effects of these factors are limited, due in part to a lack of appropriate tools. Within this program we are developing genomic information and tools for studying marine mussels (*Mytilus* spp.). In addition to being important in Canadian shellfish aquaculture, mussels are widely used as bioindicators of ecosystem health. Libraries will be generated from mussels exposed to a variety of stressing agents, producing sequence information in the form of expressed sequence tags (ESTs) and identifying genes involved in environmental stress responses. From these libraries a cDNA microarray will be developed for use in gene expression analysis, to examine the nature and magnitude of the stress response to these agents. Over the long term, these resources will be important for researchers and aquaculture managers interested in developing and improving mussel culture, as well as those utilizing mussels for assessments of coastal environmental health.



## Introduction and Background

Dramatic changes in the way that coastal socio-ecological systems are utilized have taken place in British Columbia. To ensure healthy ecosystems and resilient communities, it is necessary to improve tools for assessing environmental health that can be used to develop appropriate management methods for coastal and marine resources. The 2006 International Council for the Exploration of the Sea (ICES) report[1] identified understanding the structure, processes and biological functioning of marine ecosystems as a major goal. The need to develop ecosystem indicators specific for shellfish aquaculture and to evaluate ecosystem quality parameters and their effects on the productive capacity of coastal systems was stressed in the report. The assessment of biological effects has been found to be of great value in management terms for determining coastal water quality,[2] and therefore the productivity of marine systems.

### *Ecosystem health*

Defining ecosystem health is a dynamic process requiring identification of health indicators and how they can be used to assess overall system performance,[3]and evaluate the system sustainability as a function of activity, organization and resilience.[4] All organisms are strongly affected by their surrounding environment and environmental factors play an important part in shaping the ecology and evolution of biological systems.[5] The function of marine ecosystems can be affected by the presence of stressing agents from environmental (e.g., temperature, salinity, food availability, oxygen levels), biological (e.g., bacteria, toxic algae) and anthropogenic sources (xenobiotics, aquaculture practices, etc.). In addition, factors such as habitat degradation, overexploitation of natural resources, invasive species and pollution also affect ecosystem function. Unexplained shellfish mortalities in four major BC aquaculture companies accounted for $6 million in lost sales in 2007 alone. It is likely that the complex interaction of these factors has been responsible for the mass mortality events, but it is not known to what extent each factor contributes and what combination of factors result in fatalities.

The stress level experienced by an organism is a product of a constant or fluctuating stress and exposure time. The level of sensitivity will vary with species, population and life stage (Figure 1).

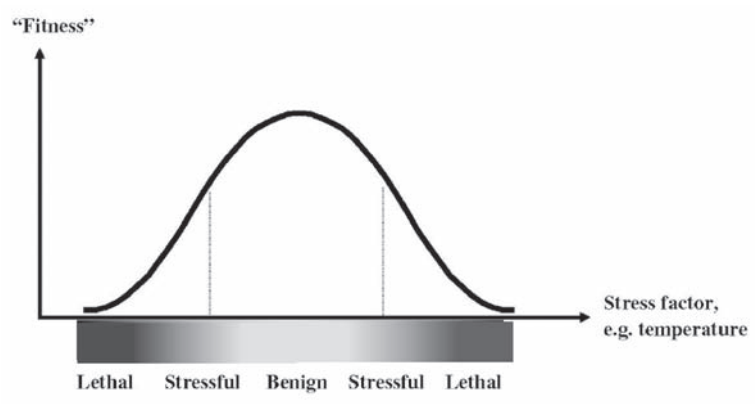
Mussels have long been recognized as valuable bioindicators of ecosystem health,[4] as described below. Ecosystems are exposed to multiple stressors[1] and one of the major problems in assessing shellfish health is determining the organism's response and the relative contribution of each parameter to stress levels. The integration of multiple disciplines (e.g., physiology, molecular genetics, evolutionary biology) holds considerable potential for major advances in the understanding of stress adaptation.[5]

### *Bioindicator and aquaculture species*

Often referred to as keystone species, bivalves are major components of coastal and estuarine ecosystems and play a prominent role in the development of ecosystem health indices and values, which can then be applied to ecosystems in general.[4] Marine bivalves of the *Mytilus* group (mussels) are dominant members of these habitats with a wide geographical distribution in both northern and southern hemispheres. They are widely studied as model organisms for various physiological, biochemical and genetic investigations with 7126 citations to date, including 2952 in biology, 876 in fisheries and 2534 in environmental sciences and ecology.[6] As targeted early exposure biomarkers of pollution, marine mussels are used in U.S. and international 'Mussel Watch' coastal health programs as they accumulate chemicals and algal toxins.[7] Their sedentary mode of life makes them accurate *in situ* bioindicators of pollution. compared to mobile species such as fish.[8] To date, most research has focused on toxin accumulation in tissues or the biological effects of pollutants by using biomarkers to reflect the physiological status of the mussel.

Mussels are economically and socio-culturally important as a tradi-

**Figure 1**
**Hypothetical relation between fitness measure and the amount of stress applied to the organism.**
**From Sørensen and Loeschcke.[5]**



tional and contemporary food source, and as part of a worldwide aquaculture industry.[9] Aquaculture is growing more rapidly than other food-producing sectors—average rate of 8.8% per year since 1970.[10] Molluscan aquaculture production presents an economic opportunity, and a better understanding of aquaculture practices and environmental contributions will create a more sustainable industry, with social and economic benefits. Three species of *Mytilus* are found in British Columbia: *M. trossulus* (native species), and *M. edulis* and *M. galloprovincialis* (introduced species). *M. edulis* and *M. galloprovincialis* form the basis of significant and successful shellfish culture industries worldwide. In British Columbia, the mussel culture industry is rapidly growing due to ambitious development programs and investment by both BC-based and international companies. A major threat to the continued success and further development of the industry is the catastrophic mortality events that have occurred in both native and non-native mussel populations. The reason for the mortality is not known, although it is thought that a combination of external and internal factors, such as fluctuating environmental conditions, reproductive status and endogenous energy reserves, play a role. If the mortalities are caused in whole or in part by these conditions, it is important that the critical factors be identified so that culture sites can be appropriately located and/or management plans developed to reduce risk. The research tools currently available have proven to be inadequate to determine the cause of these mortalities.

It is well documented that stressful environmental conditions affect physiological performance (e.g., growth and fecundity), health and survival. However in comparison to finfish, for which sensitive biochemical assays, molecular techniques and visual indicators of stress are available, there are few informative and reliable tools for bivalves. At present, the tools for bivalves include visual indicators of acute and chronic mortality phases;[1] others such as shell growth have proven to be highly variable and not always informative.[4,7]

***Genomics application to environmental health and existing bivalve genomics studies***

Genomic techniques are being widely applied in toxicology, agriculture, and aquaculture research. Improved understanding of the ability of populations to adapt to changing conditions has yielded practical benefits through recommendations to agencies making decisions on stock assessment and harvesting plans.[11] The application of genomics to bivalve biomarker studies enables researchers to assess whole-animal responses in a relatively short period of time, compared to the battery

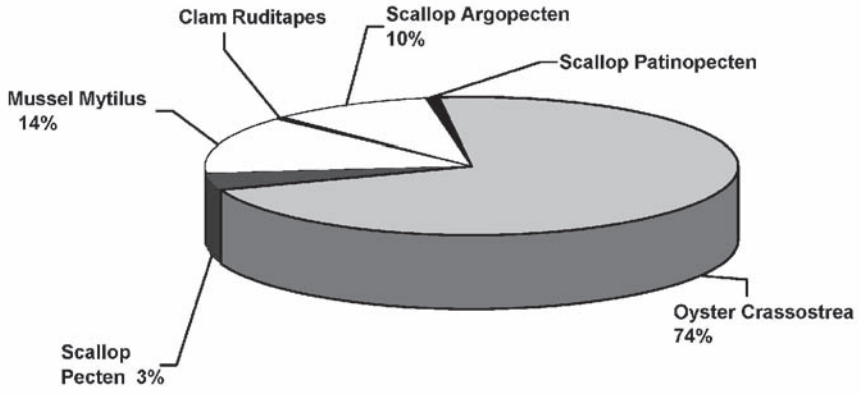
**Figure 2**
**Blue mussel *Mytilus* spp.**
**Photo courtesy of Genome British Columbia.**

of traditional tests usually employed as ecosystem indicators. These traditional techniques are end-point specific, time consuming, and individually do not provide a complete picture of the total stress response of bivalves.[5] Such techniques include histology and biochemical assays, such as those for lysosomal membrane stability, total metallothionein content,[12] glutathione peroxidase levels and the accumulation of heat shock proteins (Hsp).[13]

All physiological processes, including stress responses, are generally mediated through changes in gene expression. The identification of genes that respond to stress, such as those involved in immune responses or cellular repair, and the development of a cDNA microarray that includes these genes will provide researchers with the tools necessary to understand the transcriptional response of mussels to stress. As gene transcriptional analysis is more sensitive than measuring biomarkers at the protein level,[12] using gene expression microarrays makes it possible to identify genes that are significantly regulated by specific environmental changes in this dynamic marine system. The broader applicability of cDNA microarrays is beneficial for poorly-studied groups as they offer the possibility of cross-hybridization with closely related species, as has been demonstrated in oysters.[14] This means that this microarray will likely be suitable for the study of other *Mytilus* species and possibly other closely related species. There are still genomes of promising sentinel bioindicator species such as *M. galloprovincialis*, which have not yet been fully sequenced[15] (Figure 3). The application of microarray techniques has only recently been applied to studies of shellfish stress and health. To date such studies have primarily concentrated on a few highly valuable species of oysters,[14] and have only recently been considered for use on established integrated bioindicator[15] and aquaculture species, such as mussels.

Although bivalve genomics is currently in the early development stages, there are a variety of genomic resources for bivalves (Figure 3). With respect to *Mytlius* spp., there are at present 71,741 sequences (4253 nucleotide and 67,488 ESTs) available in public databases. These are primarily from *M. californianus* (60%), *M. galloprovincialis* (29%) and *M. edulis* (8%). The *Myt*-OME project will complement previous work on *M. galloprovincialis* and *M. edulis*, which has primarily focused on the identification of genes involved in pollution responses. To date, two microarrays had been developed for *Mytilus* species—the Mytox Array v.1.1., a low density 24-gene oligonucleotide array for *M. edulis*,[17] and a more comprehensive cDNA array for *M.* galloprovincialis, the MytArray 1.0.[18] Both were developed to examine the effects of heavy metals and hydrocarbons on gene expression, and support the development of gene expression profiling in mussels. The sequencing activities associated with the development of these arrays and the

**Figure 3**
**Bivalves in public databases. Distribution of bivalve nuclear DNA sequences (excluding microsatellites) in Genbank by species or genera. Only percentages above 1% are indicated.** Redrawn from Saavedra and Bachère.[16]



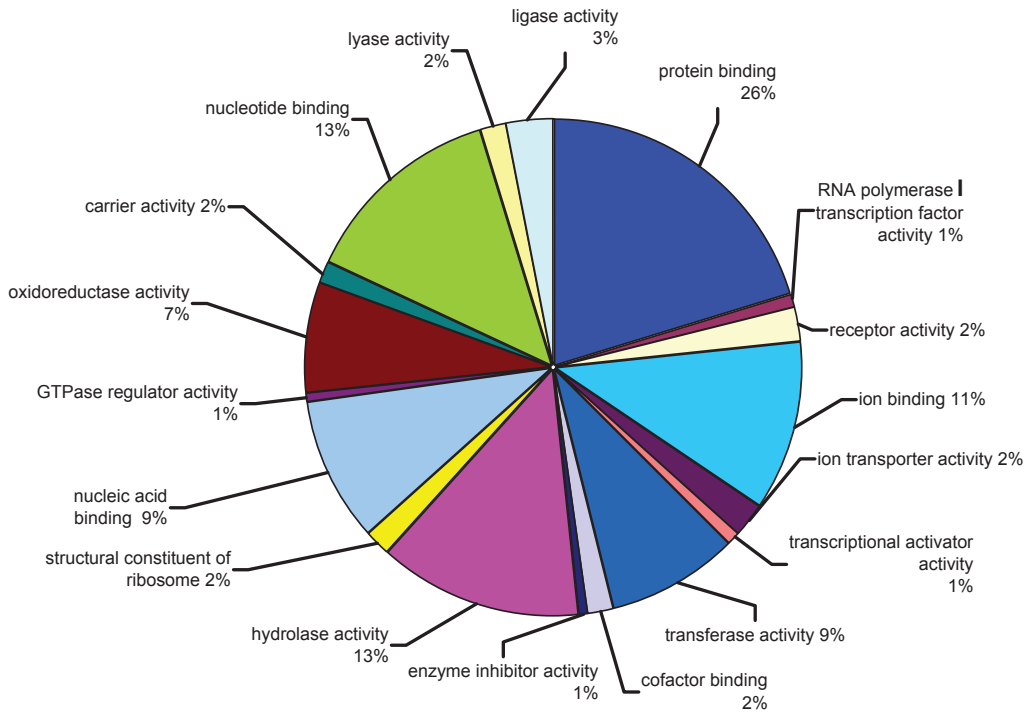
transcriptional studies that have been conducted have greatly increased our understanding of the pathways involved in heavy metal and hydrocarbon responses in mussels.[12] While these microarrays have shown that such technologies are suitable for mussels,[17-19] to date only responses to chemical pollutants have been examined. The MytArray for *M. galloprovincialis* is more extensive than the oligonucleotide Mytox array, as it consists of 1714 probes (76% singletons, ~50% putatively identified transcripts) plus unrelated controls.[18] The MytArray has been subsequently developed to include sequences specific for bacteria-associated challenges.[20] A custom array is being developed in the U.S. for the California ribbed mussel *Mytilus californianus*.[21] A 27,496 feature cDNA microarray for *Crassostrea virginica* and *C. gigas* has also been successfully developed, containing 4460 sequences from *C. virginica*, 2320 from *C. gigas* from SSH derived and unmodified libraries.[14] In addition this was developed from multiple tissues and developmental stages[22] as types of transcripts and their relative levels of expression are highly regulated by tissue specificity, developmental stage, physiological state and the environment.[15]

## The *Myt*-OME Project

### *Library and EST generation*

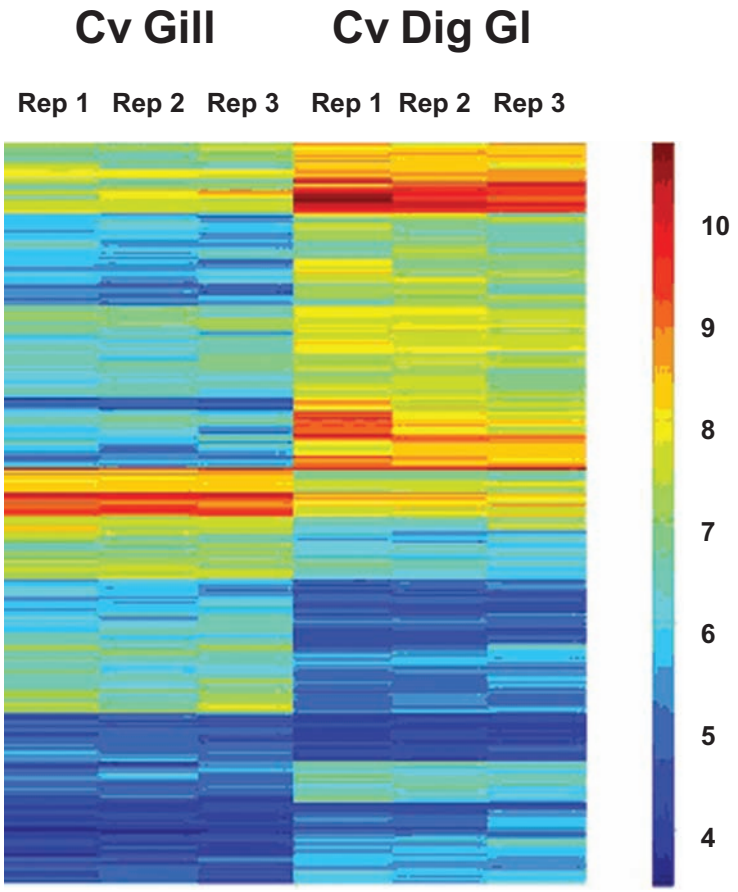
The *Myt*-OME project is designed to complement previous genomic studies on *Mytilus* spp.[17-19] This has been achieved by selecting a number of stressors not previously used in the development of libraries for *M. galloprovincialis* or *M. edulis,* [17-19] as well as partnering with other *Mytilus* genomics researchers. These stressors include: elevated temperature, hypoxia, elevated salinity, physical disturbance and desiccation and stimulation with a bacterial antigen. The *Myt*-OME project will use a functional genomics approach to identify and study genes in-

**Figure 4**
**Gene Ontology (GO) assignment of molecular function of 952 *C. virginica* annotated ESTs (3rd level GO terms). Redrawn from Quilang et al.[24]**



volved in the stress response. This approach includes the creation of targeted highly normalized and suppression subtraction hybridization (SSH) cDNA libraries, which will maximize our ability to identify stress-related genes including rare transcripts.[15] Mussels will be identified to the species level using nuclear DNA markers.[23] Normalized and SSH cDNA libraries will be produced from pooled tissues (hemolymph, mantle/gonad, gill, digestive gland and adductor mussel) and a total of 24,000 ESTs generated. Sequencing will be conducted by the BC Cancer Agency and Genome BC sequencing platform at the Michael Smith Genome Sciences Centre in Vancouver. The EST sequences will be made available through the NCBI database and as annotated sequences on a publically accessible website. Sequence annotations will be achieved using an automatic annotation pipeline based on AutoFACT.[25] This annotation tool can be customized to run a variety of similarity comparisons, and to filter the results of informative searches to generate a functional assignment for each sequence as illustrated using published data on oysters (Figure 4).[25] Bioinformatics support and website development will be provided by the Research Informatics Group at the Institute of Marine Biosciences, Halifax.

**Figure 5**
**Clustergram representing 438 genes differentially expressed in gill (left) and digestive tissues (right) from *Crassostrea virginica.* Each column represents a single array and gene expression intensity is represented by a colour-coded scale based on VSN transformed values. From Jenny et al.[14]**




### *cDNA microarray development*

Using clones generated as part of our EST sequencing, as well as clones obtained from international partners we will create a 5000 probe *Myt*-OME cDNA microarray. The microarray will be produced at the Genome BC Prostate Centre Microarray Facility at Vancouver General Hospital. As mentioned previously, we are developing a cDNA microarray as it will be more likely to be suitable for the study of other species of *Mytilus* and related bivalves. Once developed and validated, we will first use this tool to examine and solve mortality problems associated with commercial-scale mussel production in British Columbia. In addition, we will examine differential tissue responses to stressing agents (Figure 5). This microarray will also be made available to researchers outside the *Myt*-OME project where it will likely see applications in both aquaculture and environmental research, including possible routine use for long-term studies of ecosystem health and adaptability.

## Discussion and Summary

The shellfish aquaculture industry is an important economic and social component of coastal communities in BC with significant potential for growth.

However, high rates of crop mortality and peak mortality events are affecting this expansion. The use of genomics in studies of shellfish health is at an early stage and the *Myt*-OME project will significantly contribute to this research area through the development of new genomic information and tools for *M. galloprovincialis* and *M. edulis*. This project will significantly enhance the capabilities of BC in the field of shellfish genomics, aid in the development and sustainability of the shellfish aquaculture industry provincially and nationally, as well as position BC in the forefront of this research both nationally and internationally. The developed cDNA microarray will be specifically designed for monitoring ecosystem function and shellfish performance. It will provide crucial information on shellfish responses to their environment and aid in the continued development of a healthy sustainable shellfish industry through optimizing productivity and profitability, while maintaining a healthy ecosystem. This tool will also be a valuable resource to the large number of researchers studying the biology, ecology and ecotoxicology of marine mussels. The use of existing BC genomics experience and high-quality research laboratories will ensure that research quality is maintained at a high standard, and the use of the Genome BC platforms will greatly facilitate the proposed research. We plan to undertake further research into the implementation of the microarray to determine a ranking of stressing agents in shellfish. This knowledge will enable the shellfish industry to apply improved site selection criteria, and allow innovative husbandry and handling practices to improve survival rates and productivity, which will generate economic opportunities for coastal communities. This will aid in the prediction of ecosystem adaptability to changing environmental and anthropogenic conditions, and will be complemented by field work around the province. The information will be translated to the industrial sector through recommendations on aquaculture husbandry procedures, site selection, species choice and governance.

## Acknowledgements

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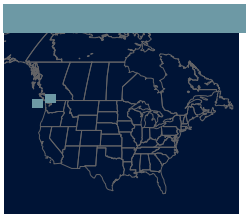

# Pilot Study of Sablefish Genomics

**Briony Campbell and Ben F. Koop**

**Ben Koop**

Sablefish (*Anoplopoma fimbria*) genomic research will provide genetic resources for a new aquaculture species as well as a valuable natural fishery. As nothing is known about the genetics of sablefish, basic resources such as polymorphic genetic markers for individual and stock identification are the most important contribution that can be made for both aquaculture and wild fishery applications. These markers enable identification, conservation and management of wild stocks, as well as broodstock selection in aquaculture. Selected matings can be based on egg quality, juvenile survival and even F1 adult production traits. Known genotypes further enable tracing fish to farm sources in commercial markets and in monitoring fish health.

Genomic studies in sablefish have three objectives:

1. To identify genes and characterise genetic variation in sablefish. Single nucleotide polymorphisms (SNP) and microsatellite markers will be developed from these genes.
2. To apply polymorphic genetic markers in:
   a. Determining population structure, migration patterns and genetic variation in wild sablefish.
   b. Monitoring and conservation of wildstocks.
   c. Characterizing individual broodstock parental genotypes in aquaculture.
   d. Initiating selective breeding strategies in aquaculture programs.
3. Development of genomic tools for general gene expression analyses with an emphasis on genes associated with growth, reproductive success, and larval metamorphosis.

The advantage of this approach is that it provides a foundation for building more extensive suites of markers for marker-assisted selection, genetic mapping of traits and disease resistance, and building gene expression microarray resources. At the same time this work provides basic, fundamental genetic tools that benefit both aquaculture and wild fisheries.



## Introduction

With 250,000 km of coastline and an Exclusive Economic Zone encompassing some 3.1 million km$^2$,[1] together with an ocean industry creating 350,000 jobs and contributing $20 billion to the economy,[2] there is no doubt that Canada is a maritime country. Its coastal communities are hubs for marine resources and/or transportation. This brings enormous responsibility for environmental safety, marine security and territorial integrity; for sustainable economic activity in coastal heartland communities; for more informed decisions by First Nation and

other coastal communities; and for baseline and integrated coastal management for British Columbia. Internationally, global aquaculture production of seafood products is forecast to increase 20 to 30 million tonnes by 2020.[3] All the projected market development will come from the aquaculture sector, leaving the commercial fishery to maintain its position in the face of shifting markets and declining natural stocks.[3-5] With a vast biophysical resource base in Canada, experience and expertise in the production, processing, distribution and marketing of fish and seafood, and coastal infrastructure to expand upon and Aboriginal opportunities and interest, Canada is well positioned to be a competitive producer of farm-raised fish and seafood.

In British Columbia, the finfish aquaculture industry is dominated by salmon. Farmed salmon is the largest agricultural export crop, with annual production of 70-80,000 tonnes and values of more than $450 million.[6] The industry directly employs more than 2,000 people in BC's coastal communities. Given the predictions for rising demand for seafood products and shortfalls of supply, there is increased interest in the expansion and diversification of new aquaculture species in BC.

### Objectives

1. To provide a genetic framework for developing a new sablefish domestic aquacultural line;
2. To provide an initial suite of genetic markers for wild fisheries management;
3. To understand and utilize exceptional phenotypic traits such as rapid growth, larval survival, and reproductive fitness.

Sablefish (*Anoplopoma fimbria)* are the most valuable species on Canada's west coast. The wild catch is currently 3,000 tonnes (< 10% of the North American total).[6] Over the last decade, demand has increased while supply has steadily declined, due to declining biomass and catch (especially in the US, which dominates the market).[7-9] In 2005, the market shortfall was estimated to be over 20,000 tonnes. At market prices over CDN$8.00 per pound, this shortfall is valued at CDN$350 million per year.[10] Given this significant opportunity, several BC companies have developed plans for farming sablefish. Three hatchery facilities—Island Scallops Ltd., Unique Sea Farms Ltd., and Sablefish Canada Ltd.—have succeeded in producing juveniles. Annual production is currently 100,000 to 500,000 juveniles and business plans are to expand this to over 2 million.[11] Developing the technologies to provide consistent production of adequate numbers of juveniles throughout the year will be essential for grow-out farms to expand into the sablefish market and provide a quality product year-round.

Wild sablefish fishery associations have strongly criticized attempts to establish farming operations on the basis of the "absence of environmental or economic studies".[12] Concerns have been raised about the role of farms as reservoirs of disease and sources of pollution, and the risk of genetic interactions, habitat destruction, and destruction of existing lucrative markets. The Canadian Sablefish Association (CSA) has called for: 1) an environmental assessment to evaluate the risk of genetic, ecological, disease and parasite impacts, 2) a socio-economic cost benefit analysis, and 3) traceability of wild and farm sources.[12-14] These criticisms highlight the limited knowledge about sablefish population structure and migration, developmental and growth characteristics, reproductive success and capacity, disease threats, ecological adaptability and many unique physiological processes. Genomic studies will provide an initial baseline of genomic resources that will enable wild fisheries and aquaculture to inform or "evolve the debate associated with sablefish aquaculture from one based on opinions to one based on evidence"—the

first recommendation of a report to DFO from the Centre for Coastal Health.[15]

Despite sablefish being a $300 million/year fishery in North America, virtually nothing is known about the genetics of the species and little is known about its biology. Over the last 42 years, 181 peer-reviewed papers[16] have included some aspect of sablefish biology. But in most of these, sablefish has been a secondary topic. Presently, there are a total of 13 DNA sequences (12 mtDNA) in public databases[17] and all were obtained from studies focusing on the related rockfish or a particular disease organism. There are no peer-reviewed published papers on genetic markers, allozyme studies or genetic population studies for sablefish.[15] Therefore, even a moderate contribution of genomic information has the potential to have an enormous impact on studies of population structure and migration, development and growth, reproduction, health and disease, ecological stress patterns, and other physiological processes. Genomic studies will provide fundamental information and resources that can be utilized in both sustainable fisheries and aquaculture.

**"Exceptionally rapid growth rates are one of the traits that make farming sablefish so attractive."**

Most information on sablefish is based on technical reports, fisheries catch data, and tagging studies.[7-,9,15,19] Sablefish inhabit the northern Pacific Ocean from northern Mexico to the Gulf of Alaska, and from the Bering Sea to Japan. Adult sablefish occur along the continental slope, shelf gullies, and in deep fjords, generally at depths greater than 200 m. The larger populations of sablefish are centered in northern British Columbia and the Gulf of Alaska. Sablefish spawn from January to March along the continental shelf at depths of 250 to 750 m and produce from 60,000 to over one million eggs. Larval sablefish are found in surface waters over the shelf and slope in the spring.[20] Juveniles are highly migratory, with significant movement from nursery areas in northern BC to the Gulf of Alaska and the Bering Sea. Sablefish move to deeper water as they mature. Sizes at maturity range from 52 to 61 cm for 5-year-old males and 58 to 71 cm for 5- to 7-year- old females. Sablefish growth is very rapid[21] for the first 3 to 5 years and slows asymptotically thereafter. Exceptionally rapid growth rates are one of the traits that make farming sablefish so attractive.

Based on tagging studies and differences in growth rate and size at maturity, sablefish are thought to form two populations.[21-23] The northern population inhabits waters off Alaska and northern British Columbia, while the southern population is in southern British Columbia, Washington, Oregon and California waters. Mixing of the two populations occurs off southwest Vancouver Island. Sablefish are managed as large homogenous populations because they are highly migratory for at least part of their life. Genetic resources are needed to determine migration patterns, genetic variation, and stock structure.

The aquaculture industry has identified clear economic benefits in de-

**Young sablefish**

veloping sablefish farming and is willing to invest substantial private funds. A major breakthrough that makes it possible to raise sablefish from eggs is the identification of conditions that permit larvae to survive through to juvenile stages capable of feeding on dry pellets. Hatcheries are able to obtain 10% survival through larval rearing—a substantial increase over rates observed in the wild—which will provides clues for understanding and perhaps improving wild fish survival.

The primary remaining challenge to developing a domesticated sablefish line is to understand reproductive maturation processes and growth factors, so that farmers can avoid early maturation in males (thereby reducing muscle production) and facilitate egg development and maturation in females. To date, mature males and females from the wild are being brought into hatcheries to serve as broodstock. This year represents the first attempt to close the domestication cycle by using selected fish raised on farms as broodstock. One of the most significant concerns in selecting breeding males and females (either from farms or the wild) is the preservation of genetic diversity while selecting for healthy and productive stock. The ability to maximize genetic diversity through the development of polymorphic genetic markers and genotyping broodstock provides one of the most important and immediately practical benefits of genomics studies. This resource not only provides aquaculture with tools to make good mate choices but also provides management tools to assess population structure and migration patterns. Markers to identify and trace products will improve food safety for export markets. On a longer term, the resources developed from this project will identify genes and markers associated with phenotypic traits of broad interest such as growth, disease resistance and health, reproductive fitness and other traits needed for superior broodstock. The development of a preliminary microarray platform will further enable biological processes like larval metamorphosis, genetic defects, and exceptional growth rates to be better understood and perhaps applied in the future as the sablefish industry grows. The very early stage of sablefish aquaculture provides an excellent opportunity to develop genetic and genomic resources from the first stages of an industry.

The Genome BC Fisheries Sector Strategy[24] has identified aquaculture—specifically, new finfish aquaculture species—as an area with opportunities. The Sector Strategy notes that "…it will be helpful to understand how potential genome science applications will impact both the fishing and the aquaculture industries…". In particular, health and disease, aquaculture, and conservation and sustainable wild fisheries are targeted for genomics research to address critical challenges and opportunities. Sablefish genomics directly targets all these objectives.

The current sablefish genomics **pilot project** is developing a preliminary suite of genomic tools to provide important fundamental contributions to sablefish biology, aquaculture and fisheries management. The novelty of this project is in providing basic, low-cost, high-impact genomic tools that will easily allow for the possibility for major future expansion. More comprehensive genetic maps, gene lists, markers and physical clone resources can be added as the industry develops.

## Research Activities

### *Gene identification (Anoplopoma fimbria)*

One of the most important initial **goals** will be to identify and characterize most of the key genes of *A. fimbria*. There are currently less than a dozen genes known from *A. fimbria* in the public databases (GenBank[25] as of December 2008) and less than 16,000 ESTs (GenBank[25] as of December 2008) from the Scorpaeniformes order (sculpins & rockfishes). Within Acanthopterygii,

Scorpaeniformes is separated from well-characterized stickleback by approximately 160 MY, and pufferfish and cichlids (Perciformes) by approximately 200 MY.[26] The estimated genome size is 0.71 pg or an estimated 710 Mb.[27] The potential relatedness of sablefish genomes to a well-characterized stickleback genome provides an important advantage with respect to annotation and determining possible function, but it remains different enough (170 MY) to require its own genetic resources. Comparative genomic technologies in salmonids[28,29] have shown how specific species information can be effectively combined with closely and more distantly related species in order to provide a more comprehensive gene order and identification. Our objective is to characterize and annotate 10,000 to 15,000 cDNA clones that will represent 8,000 to 10,000 new sablefish genes.

To accomplish this, new normalized cDNA libraries will be constructed and sequence characterisation done by Sanger sequencing of the 5′ and 3′ ends to provide a longer ~1.5 kb representation for each transcript (which means that many of genes will be completely characterized). Using data analysis systems already in place, sequence base calling, trimming, contig assembly, data submission and automated annotation will be done with existing computational pipelines.[29] EST data can be available to the public within one day of processing and submission to GenBank.

A comprehensive gene list is fundamental to all areas of genomics and proteomics, candidate gene identification, species and population analysis (microsatellite and SNP identification), and gene expression analysis. In past projects, cDNA sequence characterization has resulted in large numbers of full-length cDNAs, so we anticipate characterizing several thousand nearly complete genes. Knowledge of full or even partially characterized genes tremendously enhances RNA-mediated interference strategies that target endogenous RNA and effectively silence targeted genes. A list of genes also provides many options for vaccine development. In this regard, our close working relationship with industry will enable targets to be rapidly assessed. Identification of genes provides the foundation of all subsequent studies and is the most important initial step in the study of new genomes.

#### *Genetic variability*

A second **goal** is to provide a suite of well characterized molecular markers that can be used to examine the extent of genetic variation within and between populations of *A. fimbria* both in wild and domestic circumstances. Several types of molecular markers have been used to study population dynamics. These include allozymes, RAPDs, ribosomal RNA genes, mtDNA and microsatellites. Microsatellites are likely to be the most useful of the nuclear genome markers for

**Sablefish at 2.5 kg**

population discrimination at these first stages of sablefish studies as they generally have a large number of alleles and thus provide more information per locus than single nucleotide polymorphisms (SNPs). Indeed, microsatellites are used routinely in forensic science to identify individuals, in breeding programs to assign parentage and in population genetics to measure gene flow.[30-34] Microsatellites complement the maternally inherited mitochondrial genome sequence information to provide a more complete evaluation of the population structure and dynamics of a species.

The **plan** is to screen the *A. fimbria* EST database for microsatellites as well as SNPs, and to characterize these markers with respect to optimal annealing temperatures for PCR primers, range of alleles sizes for each microsatellite locus across the range of *A. fimbria* and the average heterozygosity of each marker. Because the markers will be associated with ESTs and genes, many of which will be annotated and differentially expressed, we will have the opportunity to identify markers and genes that participate with production traits such as growth, health and reproduction.[35, 36] Given the number of ESTs that will be produced, we anticipate being able to characterize at least 250 microsatellite loci and be able to choose the best for this project. Procedures described in Wright et al.[34] can be used to screen 25 to 100 individuals from various populations distributed across the northwestern Pacific range of the species. This allows the assessment of the average heterozygosity for each locus and so determine which markers will be most informative for further population studies. If population sub-structuring is identified, then subsequent studies with a more thorough sampling of locations will be required. For aquaculture stocks, broodstock parents can be genotyped (both current and archived samples) and samples of high and low grade mature farm fish can be examined, as well as both high and low grade hatchery juveniles.

Microsatellites and SNPs can be used to test the homogeneity of *A. fimbria* populations in the Pacific Ocean, and to determine the amount of genetic variation and its distribution within this species (i.e., is there a north and south distinction). In addition, these genetic markers allow the identification of parental broodstocks and link parents with offspring. This information enables us to maximize genetic diversity, begin selection processes for identifying for superior parents, and start the process of marker assisted selection with genes associated with traits of interest (GH, IGF, myostatin, IGFR, etc).

#### *Gene expression tools*

The **goal** is to provide a sablefish microarray platform to the general scientific and business community in order to rapidly apply genomics tools and information to areas of conservation, health and the environment. There are several formats for microarrays; however, since sablefish are not closely related to many other species, cDNA arrays that allow for cross-hybridization between species are of limited value. Large oligonucleotide (70-mers) formats provide more specific hybridization but vary with respect to the costs of on or off-slide oligo synthesis. A primary **objective** is to design and synthesize an initial ~4 to 6000 oligos (70-mers) derived from *A. fimbria* gene EST contigs and spot them on glass slides. The design of oligos and microarrays will be available to all academic investigators.

The choice of EST contigs to be represented on the array will follow the general protocols. Briefly, oligos will represent EST contigs (rather than individual ESTs) and chosen such that they differ from all others by more than 6%. They will be biased towards the 3′ end (poly A) of the contigs. Oligos from contigs for which there are at least two lines of evidence indicating a valid transcript (more than 1

EST or have a significant identity to annotated proteins) will be given priority.

Experience with salmonid microarrays is that arrays open up a multitude of experiments ranging from drug discovery and monitoring to basic biological processes. Arrays offer many investigators a reasonably well-developed method of accessing initial genomic information about their particular interests. Initial experiments will obtain preliminary data for larval metamorphosis and development as well as for precocious male development and the use of hormones to stimulate egg production. The success of early demonstrations can lead to new projects and greater resource development.

## Summary

This study initiates the development of genetic resources for a new aquaculture species as well as a valuable natural fishery. As nothing is known about the genetics of sablefish, basic resources such as polymorphic genetic markers for individual and stock identification are the single most important contribution that can be made for both applications. These markers and resources will enable identification, conservation and management of wild stocks as well as enable broodstock selection in aquaculture. As this is the first exploration of sablefish genomics, we have emphasized a very tight focus. Future work will depend on the outcome of these initial studies and the success of sablefish as an aquaculture species.

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The time has come for further interactions between academia and industry to translate the new knowledge that has been created into useful applications. To facilitate this, Genome BC's Strategic Opportunities Fund not only provides financial support to researchers, but aims to foster partnerships with national and international stakeholders in genomics and facilitate new and innovative means of interactions with industry and other end-users of genomics. This funding program is ongoing and interested applicants should visit our website at *www.genomebc.ca* for detailed information.

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