Proceedings of the
Aquaculture Biotechnology Workshop

11-12 May 2004, St. Andrews, NB

the third St. Andrews aquaculture workshop

The St. Andrews workshop series began in 2002 as a collaboration between the Aquaculture Division of the St. Andrews Biological Station (DFO) and the Aquaculture Association of Canada.

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Front cover and page 1: Designs developed by Fisheries and Oceans Canada to promote the Aquaculture Biotechnology Workshop [credit: DFO, Ottawa].

Introduction

We are living in the age of biotechnology. There is little doubt that biotechnology will fundamentally alter and improve the quality of Canadian lives. Biotechnology is already playing a leading role in transforming the health and agriculture industries in this country. The aquaculture industry is also poised to benefit from the new enabling technology to improve its productivity and competitiveness. The application of genomics will also help the aquaculture industry provide safe, high-quality, health-promoting seafood through safer and environmentally-friendly production methods. There is tremendous momentum, enthusiasm and commitment from the government to support biotechnology innovations, while making sure that adequate safeguards are in place to avoid any negative impacts to the environment and the ecosystem.

This workshop provided the forum for industry, Fisheries and Oceans Canada (DFO), National Research Council, universities, and provincial governments in the Atlantic region to discuss recent biotechnology innovations in aquaculture and the priorities the aquaculture community attaches to these developments.

The St. Andrews Biological Station (Aquaculture Division) led the workshop with the support of DFO, Ottawa and the Aquaculture Association of Canada (AAC). This is the third workshop in a series initiated in 2002 as a collaborative effort between the Aquaculture Division and AAC. The previous workshops focused on Early Rearing of Haddock—State of the Art (October 16-17, 2002; AAC Special Publication Number 7) and Early Maturation of Atlantic Salmon (March 6, 2003; Bull. Aquacul. Assoc. Canada 103-1).

The Aquaculture Biotechnology Workshop held at the Fairmont Algonquin Hotel from May 11-12, 2004 brought together more than 70 participants to focus on 3 key research areas: breeding programs, reproductive manipulation and aquatic animal health. The potentially relevant topics such as nutrition, nutraceuticals, probiotics and bioactive products from aquaculture had to be left for future meetings because of the limited time available. There were also discussions on opportunities for partnerships and the sharing of innovations and intellectual property. Speakers were invited from Norway, France and USA to provide an international perspective at this workshop.

"The Aquaculture Biotechnology Workshop . . . brought together more than 70 participants to focus on 3 key research areas: breeding programs, reproductive manipulation and aquatic animal health."

During the workshop, the open discussions on recent genomics and biotechnology advances and their impact on the improvement of seafood production and quality were very useful for the aquaculture sector to increase knowledge, exchange ideas and draw conclusions on the challenges required for the present and future of aquaculture.

The main messages from the workshop were as follows:

- Government and industry must continue to establish good traditional breeding programs and then build the genomics advances in a practical way onto these existing platforms. This was outlined for a number of marine finfish species that currently are lead candidates for cultivation. There is a need to establish species-specific broodstock programs early in the cultivation process of a species. Molecular genetics and traditional breeding programs need to be integrated to achieve gains of heritable traits from individual, family and stock selection in both the short and long term. Canada, USA, Norway and other countries should continue to discuss and combine the knowledge gained in initiating broodstock programs for marine species.

- There is a need for research on preventing or minimizing early maturation in marine finfish species. The dual benefit of sterility to enhance growth and reduce the chances that escaped fish might inter-breed with wild fish is an area of interest with direct application to the aquaculture sector. Some approaches discussed were genetic and hormonal manipulations including triploidy and all-female production. This work is being expanded from salmonids to pleuronectoides and gadoids.
- Genetic-environment interactions are important factors that require more attention. Stocks perform differently in accordance with their origin and the environment in which they are ultimately grown. Even vast differences in phenotypic performance occur among individuals within a stock. These aspects require greater consideration for all cultivated species from both an animal performance and genetic strain perspective. Care must be taken in stock selection when going forward with a breeding program.
- Canada, USA and France may wish to explore the potential collaboration on sequencing the genome of oysters because of our shared interest in this industry sector. The shellfish industry would also benefit from more studies on triploidy and tetraploidy in molluscs.
- We need to develop practical, complementary molecular tools for diagnostics and treatment of aquatic animal diseases. An effective aquatic animal health program requires many organizations to work together, to share information and to bring various disciplines together to achieve these developments.

There were 18 oral presentations and 11 poster presentations delivered during the workshop; of these 15 have been prepared for publication in this issue of the *Bulletin* to form the workshop proceedings and thereby provide a permanent record of the current effort and contributions to the rapidly developing aquaculture biotechnology sector.

—Ed Trippel and Peggy Tsang
Workshop Co-chairs

Other members of the organizing committee were: Jay Parsons, DFO, Ottawa; Christina MacGregor, AAC; Manon Chouinard, DFO, Ottawa; Susan Waddy, DFO, St. Andrews; and Steve Neil, DFO, St. Andrews.

Some of the invited speakers with the conference chairs (l to r): Ed Trippel, Peggy Tsang, Kimberly Reece, Hans Magnus Gjøen, Pierre Boudry, and Ximing Guo.

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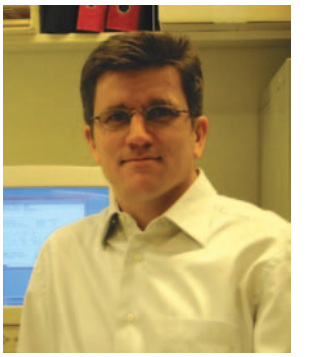

Hans Magnus Gjøen

Traditionally, selective breeding has been the method of choice for improving broodstock, both in terrestrial and aquatic animal production. Considerable improvements have been achieved for most species when sound base populations have been established and appropriate selection methods have been applied. This approach, however, is based more or less on a theoretical "black box" in terms of understanding the concrete bridge between single genes and phenotypic expression. In parallel, there is an ongoing revolution within experimental biology, and enormous amounts of data are produced. The sequencing of whole genomes has given new possibilities. For example, micro-array technology makes it possible to measure genome-wide expression levels simultaneously. In addition, genetic markers are widely developed and used. Marker-data in itself can be analysed with the traditional models to find so called QTLs. However, the traditional model of gene action, which is based on additivity, is not well suited for handling data on the levels of mRNA or proteins, as these are expressions of a dynamic system of interacting genes, rather than independent effects. Thus, new gene-regulatory models have to be developed. Traditional selective breeding will remain a "main engine" in breeding programs in the foreseeable future, but the enormous amount of new experimental data and new gene technological tools require a whole new modelling concept for further refinement of our genetic improvement efforts. This will also require specific physiological, biological and hands-on knowledge for each species, and multidisciplinary research networks.

". . . a proper selection scheme will normally create around 10% genetic gain per generation for a trait like growth rate."

Status of Breeding Technology: Traditional Selection Works

It is well documented that conventional selective breeding works well for all species tested. If inbreeding is avoided and the population has sufficient genetic variation, a proper selection scheme will normally create around 10% genetic gain per generation for a trait like growth rate. Selection response is accumulated each generation, so considerable accumulated response can be generated if we work patiently.

The main principle in traditional quantitative genetic theory is the infinitesimal genetic model and the concept of additivity of genetic effects. The infinitesimal genetic model assumes that there are an infinite number of genes behind a quantitative trait, whereas the principle of additivity assumes that all genetic effects, as well as dominance and epitasis, can be modelled independent of each other. We will come back to the shortcomings of these assumptions later, but the fact is that

despite its simplicity, these models have proven to be very powerful and useful when describing, simulating and planning a breeding program.[1,2]

When simulating any breeding program, it is customary to generate about 15 generations to clearly display the long-term effect of selection. The most important parameter to monitor carefully is the rate of inbreeding, which should be kept at an acceptable level. A rule of thumb that is often applied and cited is to at least keep the effective population size (Ne) above 50, which corresponds to a rate of inbreeding of 1% per generation. By using these tools, it is possible to tailor-make breeding schemes to fit biology, resources and facilities at hand.

The final goal for any breeding program would be to maximize the genetic gain for the trait of interest. Genetic gain is generally expressed by:

$$\mathbf{G} = i \quad_{G} r_{GI}$$

where

- i is the selection intensity,
- $_{G}$ is the true genetic variation,
- r_{GI} is the correlation between the true genetic value and the estimated breeding value, often called the accuracy of the breeding value estimate.

"The most important parameter to monitor carefully is the rate of inbreeding . . . A rule of thumb . . . is to at least keep the effective population size (Ne) above 50, which corresponds to a rate of inbreeding of 1% per generation."

The difficult task of any breeder is to optimise these parameters, since none of them can be easily enlarged without negatively influencing one or both of the others. This can be adequately done through simulation studies as described above, and this will be an important tool for breeders in the future too.

Immediate Potential for New Gene Technology

High expectations have been brought forth concerning the results that new gene technology can achieve for the benefit of animal and fish production.[3] This has caused public and private bodies to spend millions of dollars on finding vital genes for economically-important traits. Sad to say, many of these projects have given few results, and this might lead some to the conclusion that gene technology is still a futuristic adventure. However, there are some immediate applications for the technology. These may be categorised as 1) improvement of traditional systems, and 2) surpass of "difficult" traits.

Improvement of Traditional Methods

An efficient breeding program for fish requires the possibility of family identification. The main drawbacks with conventional breeding schemes and tagging methods are the introduction of common environmental effects prior to tagging, high costs related to establishing and operating facilities for separate family rearing, and limited number of individuals tagged within each family.

These drawbacks can all be solved by using DNA-typing as a tagging system. This allows for:

- communal rearing of individuals from many families in the early life stage,
- minimal need for special facilities for separate family rearing,
- utilisation of the enormous display of Mendelian sampling that is represented within the large fullsib groups produced by most fish species.

Towards the end of the growth period, the required number of the largest fish are DNA-typed and marked with a physical tag. In addition, other traits can be efficiently recorded by using smaller parallel lines with all families represented in fewer and standardised numbers. Here all fish are recorded and typed. With appropriate software, pedigrees can be established, and the breeder is able to estimate high accuracy breeding values through use of BLUP.

Simulation studies and preliminary growth results from tilapia have shown that this technology can improve the efficiency of selective breeding by at least 10-20% compared to conventional schemes.

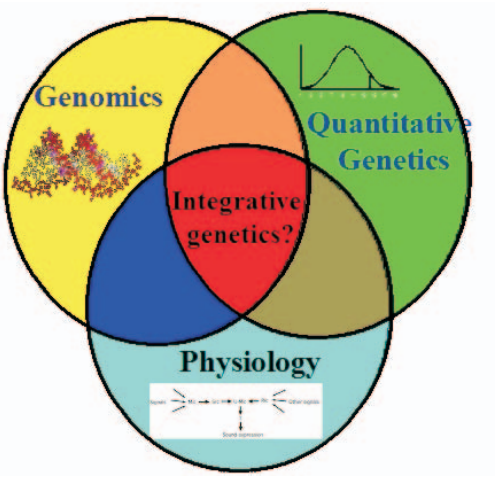

Figure 1
New technology and knowledge allows the merging or linking of sciences that previously did not interact efficiently.

Surpass "Difficult Traits"

Many traits important for economical fish farming, like disease resistance, fillet colour and fillet quality, are not measurable in live individuals and thus not obtainable for the breeding candidate itself. For such traits, the breeder is dependent on other sources of information, with fullsib records being the most important. Even though this is a valuable breeding method unique to fish, it only allows us to utilise half of the genetic variation present for the trait. Individual records would allow full utilisation of the genetic variation. One way to do this is obtain QTL-information. This is why emphasis in QTL-scans has been put on these traits and is more likely to make important contributions to multiple-trait breeding programs. Most of these traits have never been subject to selection before, and selection based on QTL-information is thus likely to be more efficient.

A New Era—Opening the Black Box between Phenotype and Genotype

There is an ongoing revolution within experimental biology as enormous amounts of data are produced from various new technologies. The sequencing of whole genomes has given new possibilities for collection of system-wide biological data. Genes can be identified within a sequence and micro array technology makes it possible to measure genome-wide expression levels simultaneously. Furthermore, differences at single nucleotides between individual sequences, SNPs, are a new class of genetic markers that give us even more powerful ways to fine-map the genomes of farmed species. At present, nearly 1.8 million SNPs have been detected in the human genome. Analysing the amounts of information that can be produced from such experiments represents a huge challenge for modern biology. SNP data in itself can be analysed with the methods of genetics. As genetic markers, the SNPs can be arranged linearly in linkage groups, and recombination fractions can be estimated to make dense genetic maps. These genetic maps can then be used in traditional genetic studies, such as the search for QTLs.

However, the traditional model of gene action, which is based on additivity, is not well suited for handling data at the levels of mRNA or proteins, as these are expressions of a dynamic system of interacting genes rather than independent effects. Several studies show that the effectiveness of marker-assisted selection (MAS) is not as efficient as previously thought (e.g., Melamed et al.[4]) and there is a need for new models that enable us to utilise vast amounts of data in a better and sustainable way. New models are also needed to mimic the true nature of genetic interaction effects, like dominance and epistasis, where conventional additive models have shown to be inadequate.

New technology and disciplines often require new models and methods to communicate the new concept in a proper way. This new area is now being developed in the union of at least three major sciences: genomics, quantitative genetics and physiology/biology (Fig. 1). Many terms have been used in relation to this new area (e.g. systems biology, bioinformatics or computational biology), but the

term "integrative genetics" tries to encompass the fact that we are on the edge of integrating all biological observations and systems down to the genetic building blocks. We are about to bridge the gap between genotypes and phenotypes by:

- integrating experimental and theoretical approaches;
- integrating processes and mechanisms connecting genotypic data with phenotypic data in a coherent mechanistic explanatory structure; and
- applying the explanatory frameworks of non-linear system dynamics and statistics.

This will typically include the use of positive and negative feedback-loops to describe the way that the expression of genes interact, in contrast to simple linear manners in conventional models. This new approach has also shown to be very efficient in describing genetic effects like dominance and epistasis.[5]

One other important aspect of this approach is that it will allow us in a new way to link phenotype (P), genotype (G) and environment (E) in a more realistic way. Conventionally, we used to limit our models to threat these factors in a simple additive way P = G + E. But genetic and environmental aspects have to be considered jointly, not only as an interaction element in the conventional equation (e.g. P = G + E + GxE), but as a dynamic system allowing for the non-linearity described above.

". . . we are on the edge of integrating all biological observations and systems down to the genetic building blocks. We are about to bridge the gap between genotypes and phenotypes . . . "

Describing such systems completely is likely to be extremely complicated for composite traits—like growth rate—but by using adequate methodology it is doable,[6] especially for more explicit traits like colouration.[7,8] For instance, recently a larger project to accurately describe and model filet colouration in salmon was initiated in Norway at the Centre for Integrative Genetics (CIGENE). Generally, the new tools require and enable multi-disciplinary approaches that will lead to an increased need for networking among scientists.

Conclusion

Traditional selective breeding will remain a "main engine" in breeding programs in the foreseeable future. Some immediate benefits of using new gene technology should be harvested. The enormous amount of new experimental data and new gene technological tools require a completely new modelling concept. Utilisation of this new gene-toolbox gives great opportunity for integrating increasingly larger amount of data and insight from biology, physiology and genetics to the benefit of a growing aquaculture industry.

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Appendix:

An Update on Status of Marine Breeding programs in Norway

On request from the organizers of the Aquaculture Biotechnology Workshop, a schematic overview of the present status of Norwegian breeding programs in marine species is given.

Atlantic cod

- ***National breeding program in Tromsø***
  - ✓ Started up in 2003, with 24 families surviving up until transfer to sea; natural spawning, 1 female to 1 male. In 2004, 100 families will be produced, but due to facility constrains, 84 will be start fed. Spawning by stripping. Both coastal and Arctic origin.
  - ✓ Successful challenge testing for bacterial disease, *Vibrio anguillarum*. Also plan to do challenge testing for virus on smaller fish. Will use DNA-typing as a supplementary system.
  - ✓ The goal is to have at least 50 families transferred to sea.
  - ✓ Breeding goal: growth and disease resistance.
  - ✓ Candidate traits: quality traits and late sexual maturation.
- ***Marine breed***
  - ✓ 50 families produced in 2001 (as a result a publicly-funded project). Produced by natural spawning, 1 female to 1 male. PIT-tagged at about 10 grams. Testing both coastal and Arctic cod.
  - ✓ Transferred to four different sea sites, from Tromsø in the north to Bergen in the south. Heritability estimates on growth up until tagging size (PIT) is estimated to be 0.3.
  - ✓ 2nd generation to be produced in 2005.
- ***Other commercial operators***
  - ✓ Cod Farmers Ltd. (earlier *Gadus*): DNA-based family program.
  - ✓ Cod Culture Norway, CCN (Nutreco): Strains/groups of different origin are kept separate.

Atlantic halibut

- ***National program in Bodø***
  - ✓ Only a few families produced in 2003, and about 25 families in 2004. The main breeding population will be DNA-typed to provide pedigree information, but families will be kept separate until major mortality has ended. There will also be set up special schemes with PIT-tagged fish for estimation of genetic parameters.

Pierre Boudry

Genetic Variability and Selective Breeding for Traits of Aquacultural Interest in the Pacific Oyster *(Crassostrea gigas)*

Pierre Boudry, Lionel Dégremont , Nicolas Taris, Helen McCombie, Pierrick Haffray, and Bruno Ernande

The most significant genetic improvement for production of Pacific oyster *(Crassostrea gigas)* has been obtained through the breeding of triploids, especially since the development of tetraploids. Quantitative genetics studies suggest that significant gains, for disease resistance or for other traits of aquacultural interest, could be obtained using this approach. However, the limited extent of hatchery-propagation (versus natural recruitment) and/or various technical difficulties and biological characteristics of the species have slowed the development of selective breeding programs. Recently, in the USA, Australia and New Zealand, family-based selective breeding programs have been initiated to improve growth and yield. In Europe, where both natural and hatchery-propagated spat are farmed, no large-scale selective breeding programs have been initiated. However, special attention has been paid to "summer mortalities", for which the causal factors are still unclear. Our studies have shown that family-based selective breeding can improve spat survival, with no impact on growth. However, a genetic trade-off between survival and reproductive allocation was shown in adults, but was influenced by environmental variation. This might explain how additive genetic variance for fitness-related traits is maintained in wild populations. Practical difficulties in breeding large numbers of families are a major constraint for family-based selective breeding in oysters. Genetic variability exists for several larval traits, which increases the imbalance in reproductive success between breeders in hatchery-propagated populations. Multiplexed-microsatellite markers can be efficiently used to trace parentage in mixed-family breeding programs. Finally, a new means of introgression of traits of interest from genetically improved diploids to polyploids will allow the combination of selective breeding and polyploidization.

"Aquacultural production of the Pacific oyster *Crassostrea gigas* is of increasing economic importance, but little genetic improvement has been made on this species . . ."

Introduction

Aquacultural production of the Pacific oyster *Crassostrea gigas* is of increasing economic importance, but little genetic improvement has been made on this species to date (see Sheridan[1] for review). In many countries, wild spat is collected and raised, which offers little or no possibility of controlled genetic improvement, although natural selection might contribute to the adaptation of stocks to local environmental conditions. This effect could be more pronounced in countries where

C. gigas was recently introduced for aquaculture and is now successfully established (e.g., Australia, France, New Zealand). However, such local adaptation is poorly documented, probably because of the high plasticity of oyster species and the difficulty of setting up comparative experiments between introduced and native stocks (but see Soletchnik et al.[2]). Furthermore, temporal and spatial variation, natural and aquaculture-induced gene flow and large effective population sizes (but see Li and Hedgecock[3]), are likely to make natural adaptation rather slow. Natural selection will primarily act on fitness-related traits, but it will not always work in favour of aquaculture. Trade-offs between growth, survival and reproduction can lead to shifts in resource allocation strategies from survival to reproductive effort and growth when resource abundance increases.[4] In such cases, natural selection would favour genotypes with low survival but high growth and reproductive allocation, and this might not be the best selective direction for aquaculture.

Triploidy as a "Single-Step" Genetic Improvement

The most significant genetic improvement for the production of Pacific oyster to date has been obtained through the breeding of triploids. Polyploid *C. gigas* have become increasingly important following findings that they grow quicker than their natural diploid counterparts, probably due to their high level of infertility.[5] This is a clear demonstration that traits which are counter-selected in the wild (such as infertility) can be beneficial for aquacultural production of oysters. The development of tetraploid oysters has eased the production of triploid oysters, now bred by crossing diploid females with tetraploid males.[6] Additionally, these "natural" triploids show better growth and survival than chemically induced triploids (see Eudeline[7]). However, triploidy is a "single-step" genetic improvement and further genetic improvement should be considered.

Field testing of the "Morest" families in the Marennes-Oléron Bay (France).

Family-based Selective Breeding: Present and Prospects

Many quantitative genetics studies suggest that significant heritable variation exists for traits of aquacultural interest in oysters, such as disease resistance or growth (for review see Dégremont[8]). Additionally, heterosis and the use of non-additive genetic variation have also been investigated[9](see also http://hmsc.oregonstate.edu/projects/wrac/). Consequently, significant gains should be obtained by selective breeding in diploid oysters. However, the limited extent of hatchery-propagation (versus natural recruitment), some technical difficulties, and certain biological characteristics of the species, have retarded the development of selective breeding programs. Until recently, most hatcheries ei-

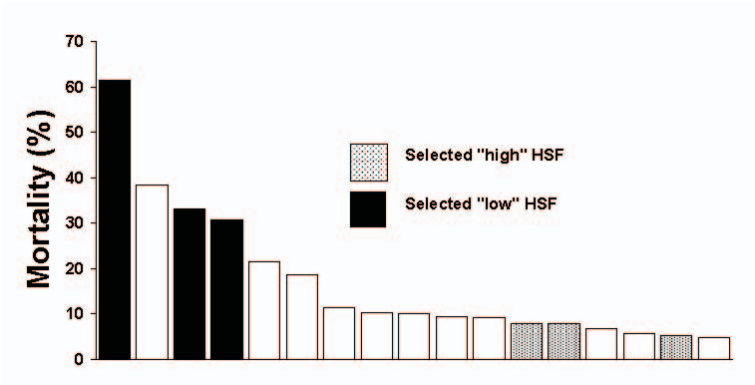

Figure 1
Variation of mean mortality among G1 half-sib families (HSF) and identification of the selected "high" and "low" families for the breeding of the second generation ($P < 0.001$).

ther chose their breeding oysters from within commercial batches or else bred from offspring that were selected by mass selection, most often on weight and morphology. Recently, in the USA, Australia and New Zealand, countries where hatcheries are a major source of *C. gigas* juveniles, family-based selective breeding programs have been initiated to improve growth and yield [10,11] (see also http://hmsc.oregonstate.edu/projects/mbp/).

In Europe, where both natural and hatchery-propagated spat are farmed, no large-scale selective breeding programs have yet been initiated. However, special attention has been paid to the role of genetics in "summer mortalities", for which the causal factors are still unclear. A national multidisciplinary program, named "Morest" (see http://www.ifremer.fr/francais/defis/defi6/morest.ht_Hlt73855766m _Hlt735855766) and coordinated by IFREMER, was initiated in France to help understand the causes of the summer mortalities in *C. gigas* and to find possible solutions.

As a first step, 15 families (5 males crossed with 3 females each, following a nested half-sib design) of one-year-old oysters were studied under controlled (IFREMER's facilities in Bouin) and natural (farming site close to La Rochelle) rearing conditions, showing contrasting food availability. The aim was not only to estimate genetic variability of survival, growth and reproductive allocation, but also the genetic component of their plasticities, giving a highly informative combined data set.[4] Phenotypically, high food abundance led to lower survival but higher growth and reproductive effort. Heritability estimates were low for growth and its plasticity, but high for survival and plasticity of reproductive effort. Interestingly, estimates of genetic correlations between reproductive effort and survival changed sign according to food abundance (they were positive when food availability was high and negative when it was low). These results clearly illustrate that multi-trait approaches should be favoured to better understand genetic variation for resource allocation traits in oysters. Trade-offs and environmental influence on genetic correlations might explain why additive genetic variance for fitness-related traits is maintained in wild populations subjected to spatial and temporal variations. However, this also makes it difficult to predict correlated response to selection for a given trait.

The second step, in 2001, concerned 72 bi-parental families ("G1"), produced in 3 sets of 24 (for each set, 6 males were crossed with 4 females each, following a nested half-sib design).[8] Due to variable larval survival and settlement success, heritability estimation of spat survival in the field during the first summer was determined using only 17 half-sib families (corresponding to 43 full-sib families). Families were monitored at 3 sites (in Normandy: Baie des Veys, in Brittany: Rivière d'Auray, and on the Atlantic

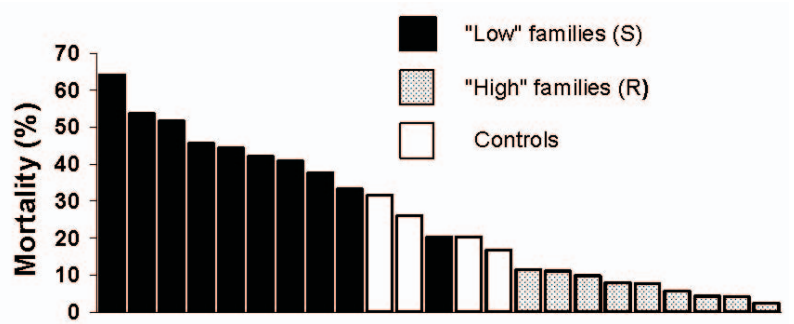

Figure 2
Variation of mean mortality in the outbred G2 families (divergent selection) in Brittany ($P < 0.001$).

coast in the Marennes-Oléron bay: Ronce), previously known to show different survival rates for juvenile oysters. A variance component analysis revealed that among the studied factors, 45% of the observed variance was due to variation among families. The heritability estimate for survival during the first summer was remarkably high. To confirm this result and to assess whether selection could be efficient, a second generation ("G2") was produced in 2002 by breeding the best and worst G1 half-sib families (Fig. 1) in order to evaluate responses to divergent selection. Both within (inbred) and between (outbred) family crosses were performed using G1 oysters that were protected from field mortality pressures (i.e., selection was strictly at the family level). Results confirmed the high heritability of survival of spat over their first summer. "High-selected" families (named "R" for "resistant") showed much higher survival than "low-selected" ones ("S" for "sensitive"). For example, in Rivière d'Auray (Fig. 2), summer mortality of outbred G2 "S" oysters was 43%, while it was only 7% for "R" progenies (the unselected control was intermediate: 24%). Similar results were found in 2003, in the third generation ("G3"), which replicated the outbred G2s without making any further selection. In Rivière d'Auray, summer mortality of G3 "S" oysters was 73%, while it was only 27% for "R" progenies (the unselected control was intermediate: 48%). In 2003, triploid progenies were also produced by crossing unselected tetraploid males with G2 "R" or "S" diploid females. Triploid "S" showed a higher mortality (58%) than triploid "R" progenies (36%), while the triploid control was intermediate (50%). Interestingly, no correlated response was observed between growth and survival in spat. Additionally, survival over the second summer was similar for "R" and "S" G1 and G2 progenies in Ronce and Rivière d'Auray, indicating that the first summer is the critical sensitive period. However this remains to be confirmed in Normandy, where mortalities mostly affect adult oysters.

These families are currently being studied to investigate their genetic, physiological and immunological characteristics further, as part of the multidisciplinary research project "Morest". Special attention is being paid to reproduction and resource allocation, which are not as easy to study on spat compared with larger oysters. Consequently, molecular approaches are being developed.[12]

Mixed-Family Approaches: Feasibility and Constraints

These results and others using similar approaches are encouraging and promising for the development of selective breeding in oysters. However, practical difficulties in breeding large numbers of families are a major constraint for family-based selective breeding in oysters. Mass selection can be considered as a more practical approach (e.g., Naciri-Graven et al.,[13] Nell et al.[14]), but genetic variability of the selected populations should be carefully monitored to ensure long-term improvement and limit inbreeding. Indeed, many studies have demonstrated that hatchery-propagated bivalve stocks have low effective population sizes,[15] which is commonly due to having a limited number of (highly fertile) parents (e.g., Launey et al.[16]). This can be easily overcome in species where reproduction and crossing are fully controlled. However, other aspects, such as density effects, are not so easy to control.

Our studies have shown that genetic and non genetic components exist for several larval traits, which increases the imbalance in reproductive success between breeders in hatchery-propagated populations at early stages. Firstly, sperm competition at fertilization tended to increase the imbalance in reproductive success between males.[17] Consequently, separate pair mating prior to common larval

rearing should be favoured to maximise effective population size at early stages. Secondly, a family-based study allowed the estimation of breeding value and genetic correlations for several early life-history traits.[18] Larval survival, development rate, size and success at settlement showed significant heritable variation. Negative genetic correlations ("trade-offs") were observed between development rate and success at settlement. Consequently, common practices in oyster hatcheries, such as selective sieving during larval rearing and at settlement (i.e., discarding the smallest and/or slow developing larvae) are likely to be significant selective pressures in hatchery-propagated stocks.

As individual tagging is impossible at early stages of life, marker-based parentage analysis of mixed families is likely to ease the evaluation of such phenomena. In order to reduce costs associated with genotyping, we have developed a set of multiplexed-microsatellite markers[19] that has been used to monitor the effect of sieving (i.e., selection of fast growing larvae) on the genetic composition of a hatchery-bred population. As expected, selective sieving of fast growing larvae leads to earlier and less variable time to settlement. More interestingly, this also leads to increased imbalance in reproductive success and, consequently, to reduced effective population size.[19] Similarly, effects of environmental conditions (e.g., temperature and food availability) during larval development are currently being investigated.

Such marker-assisted parentage analysis could also be of great use in mass selection breeding programs, in order to optimise management of the genetic resources by the minimising inbreeding or to evaluate genetic parameters on the progeny reared in a common environment.[20,21]

From Selected Diploids to Selected Triploids?

It is likely that triploidy will remain a major and expanding genetic improvement for oyster farming in the future. However, the incorporation of polyploids makes selective breeding programs more complex and slower compared to diploids. Therefore the use of polyploids might delay their contribution to the sustainability and the development of oyster farming. Furthermore, selection on tetraploids is also restricted because strict quarantine conditions must be observed for their culture in some countries. It is therefore unlikely that selective breeding of tetraploid oysters will efficiently contribute to the development of selected stocks in the near future.

10-month-old oysters from selected families.

Improved 4n lines would, however, be an important step towards the production of genetically improved triploids. It is therefore necessary to have a viable means of generating tetraploids de novo from improved 2n stocks or, alternatively of integrating genetic material from 2n animals into existing 4n oyster lines. Using eggs from 3n females relies on the fertility of these maternal genitors and the quality of their

gametes. The character of fertility in 3n animals is not desirable in aquaculture and the deliberate selection of fertile 3n to supply parents for 4n stocks may represent a problem of fertility in subsequent 3n progeny of the 4n thus produced. Indeed there are already indications that the characteristic of high fertility can be passed from triploid to tetraploid.[6] An alternative means of generating tetraploids in bivalve mollusks is directly from diploids by inhibiting the expulsion of both polar bodies.[22] The use of diploids would allow animals grown and selected under normal culture conditions to be converted directly into tetraploids. However, direct induction from diploids has generally shown low percentages of tetraploids produced per cross and low survival.[22,23]

Another means of exploiting genetic characters from diploids in tetraploid lines would be the introgression of this material into 4n families using a recently developed technique.[24] This method produces tetraploid *C. gigas* by cytochalasin B inhibition of polar body 2 expulsion in diploid females crossed with tetraploid males. This offers a means of introgression of genetic characters directly from selected diploid to tetraploid lines, avoiding a triploid step. Viable tetraploids were found at 4 and 6 mo using this method, indicating that the technique is successful.

"Improved 4n lines would . . . be an important step towards the production of genetically improved triploids."

Conclusion

Multiple directions exist for progress in genetic improvement of oyster stocks, be these via family or group selection. Molecular tools, which will be developed alongside future selection programs, represent a manner of continuously monitoring the progenies issued from any chosen path of selection and can therefore represent an aid in decision making. In practical terms, diploid oysters are the simplest to work with, even if the final objective is integration of traits of choice into commercialised polyploids. Future work should also include an evaluation of the relevance of selection on diploid progenitors relative to performance in future polyploid offspring. The choice of traits for improvement and manner and age of selection in any program should be made in the light of knowledge about the correlation structure of life history and resource allocation traits that has recently been found.

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"The choice of traits for improvement and manner and age of selection in any program should be made in the light of knowledge about the correlation structure of life history and resource allocation traits . . . "

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Use of Molecular Genetic Markers in Atlantic Halibut Broodstock Management

Debbie Martin-Robichaud

Debbie Martin-Robichaud, Darrin Reid, Tim Jackson and Mike Reith

Atlantic halibut hatcheries in the Canadian Maritimes have recognized the need to monitor inbreeding and genetic variability as they begin selecting F1 broodstock for use in breeding programs. Genotyping of 145 F1 1996 year-class fish using five microsatellite markers allowed pedigrees to be determined and family groups identified. Within this group of F1 fish, 53% belonged to 4 full-sib families, 89% to 6 maternal half-sib families and 80% to 5 paternal half-sib families. Only 36% of the crosses contributing to this group were represented in the retained offspring. The allelic variation lost between wild and F1 fish was 26%. Reciprocal transfers of fish between the four hatcheries have restored the allelic variation to approximately the levels in the parental stock. A genotype-based pedigree system is now available for industry use.

Progress has been made on the development of the first linkage map for Atlantic halibut. Approximately 75 microsatellites, as well as 12 from the Japanese flounder, have been genotyped in two maternal half-sib families. Sex-specific linkage mapping has identified 16 linkage groups in the female and 18 in the male, resulting in 19 unique linkage groups. Recombination between common markers is slightly higher in the males than the females; however a G-test reveals the difference is not significant. Morphometric data were collected for each experimental family to identify quantitative trait loci (QTL) affecting pigmentation, body size (length, width, weight) and eye migration. Eye migration (measured in degrees) and myotome height are being used as an indirect measure of halibut metamorphosis. Preliminary analyses indicate that QTL can be identified for these characteristics.

Introduction

Intensive genetic selection of agriculturally-important species has significantly improved yields in animals such as cattle (increased milk production from 2000 to 10,000 L/yr) and broiler chickens (decreased time to market from 120 to 42 days).[1] The identification of quantitative trait loci (QTL) in animals and plants has enabled the application of marker-assisted selection (MAS) programs to significantly improve the speed and intensity of selection.[1] QTL are genetic markers associated with regions of DNA (i.e., genes) that influence traits of economic importance (e.g., growth rate, feed conversion, spawning time, temperature tol-

erance, and disease resistance). While such genetic selection results in enhanced performance, the genetic consequences are decreased genetic variability and the potential for the appearance of deleterious effects due to inbreeding. Genetic selection programs thus need to balance the selection of desired traits with the maintenance of genetic diversity. Ideally, awareness of maintaining this balance begins early in the domestication of a species, before genetic variability declines.

Most cultured fish originate from essentially wild stocks and have undergone little directed genetic selection. The potential for economic gains through genetic improvement is thus largely untapped. Marine fish should have greater selection potential than agriculture stocks due to higher genetic variability and greater fecundity. However, due to economic constraints, genetic variability is often ignored during the establishment of a new fish species in aquaculture. Frequently, facilities for holding sufficient numbers of broodstock to maintain genetic variability are inadequate. Although hatchery managers know the importance of broodstock management and genetics, the first focus is on economic survival and mass production of products. Selection of the first generation of domesticated broodstock is often based on a few parameters such as growth, coloration, or even just survival during the first years of hatchery operation. However, this first selection of broodstock can have devastating effects on the industry in the long term if genetic factors are ignored. For example, a single generation of inbreeding in channel catfish resulted in slower egg development and decreased hatchability, and reduced survival and body weight.[2]

"Mass rearing of yolksac larvae in silos at appropriate biomass densities and the need to grade small juveniles makes it unrealistic to rear separate families. Consequently, the use of molecular markers for pedigree identification is particularly useful."

Molecular genetic markers such as microsatellites (which are used in DNA "fingerprinting" methods) are useful tools for evaluating genetic variability. In addition, they can be used to generate genetic linkage maps, which are a necessary tool for the identification of QTL. As well, they can be applied to an additional problem that arises due to the fecundity of fish: the difficulty of identifying family members and determining pedigrees. Many marine fish produce large numbers of small, undeveloped larvae with long early-life history stages. Mass rearing of yolksac larvae in silos at appropriate biomass densities and the need to grade small juveniles makes it unrealistic to rear families separately. Consequently, the use of molecular markers for pedigree identification is particularly useful.

The Atlantic halibut *(Hippoglossus hippoglossus)* is a large, long-lived, iteroparous flatfish that is currently being developed for aquaculture in several countries. We have previously identified potential genetic limitations in the first generation of fish selected as broodstock.[3] We report here on our efforts to minimize these limitations and our work toward developing a genetic linkage map and QTL, with the ultimate goal of applying MAS to this species.

Methodology

Fin clips were taken from 145 cultured F1 halibut that originated from one production run at a hatchery in 1996. The fish were subsequently distributed to three hatcheries in the Canadian Maritimes. In addition, the parental wild broodstock and other captive wild broodstock, all of which were captured from the Bay of Fundy, were sampled (total n=52 and parental group n=27). These samples were used for the pedigree and genetic diversity evaluation. DNA extraction, genotyping and data analysis were done as described by Jackson et al.[3] One hundred 1997 F1 from a fourth hatchery were also genotyped. Based on allele frequencies, 87 fish were redistributed between the four sites to increase genetic diversity in the F1 broodstock.

For the genetic map and QTL development, three maternal half-sib families

were reared together through first feeding and weaning at Scotian Halibut Ltd. in 2003. Near the time of weaning (61 days post first-feeding), a sample of 323 fish was taken from one of three first-feeding tanks by dipping a net from the tank bottom to the water surface. This method sampled settled fish as well as those in the water column, providing a wide phenotypic distribution for QTL analysis.

For morphometric measurements, two digital photographs were taken of 323 fish, one head-on view and one side view (Fig. 1). From the side view, length (mm), width (mm) and myotome height (mm) were measured. Simple PCI v4.0 Imaging Software (Compix Inc.) was used to quantify the percent surface area pigmented (Fig. 1). Eye migration was measured in degrees from the head-on view using Photoshop 7.0 (Adobe Sysytems Inc.).

Microsatellite markers for Atlantic halibut were previously developed in our lab.[5] Samples from two of the three mapping families were genotyped with both Atlantic halibut microsatellites and microsatellites from Japanese flounder *(Paralichthys olivaceus)*.[4] Pair-wise recombination estimates and logarithm of odds ratio (LOD) scores for all marker pairs were calculated using LINKMFEX v.1.6.[6] Marker pairs were then assigned to linkage groups at a LOD threshold of 3.0 ($p < 0.001$). Within each LOD 3.0 grouping, marker order was determined using a nearest neighbor approach. Map distances were represented in cM where 1% recombination between loci equals 1 cM in map distance (Fig. 2). An exploratory quantitative trait loci (QTL) analysis was conducted using the QTtest module within the LINKMFEX package.[6]

Results

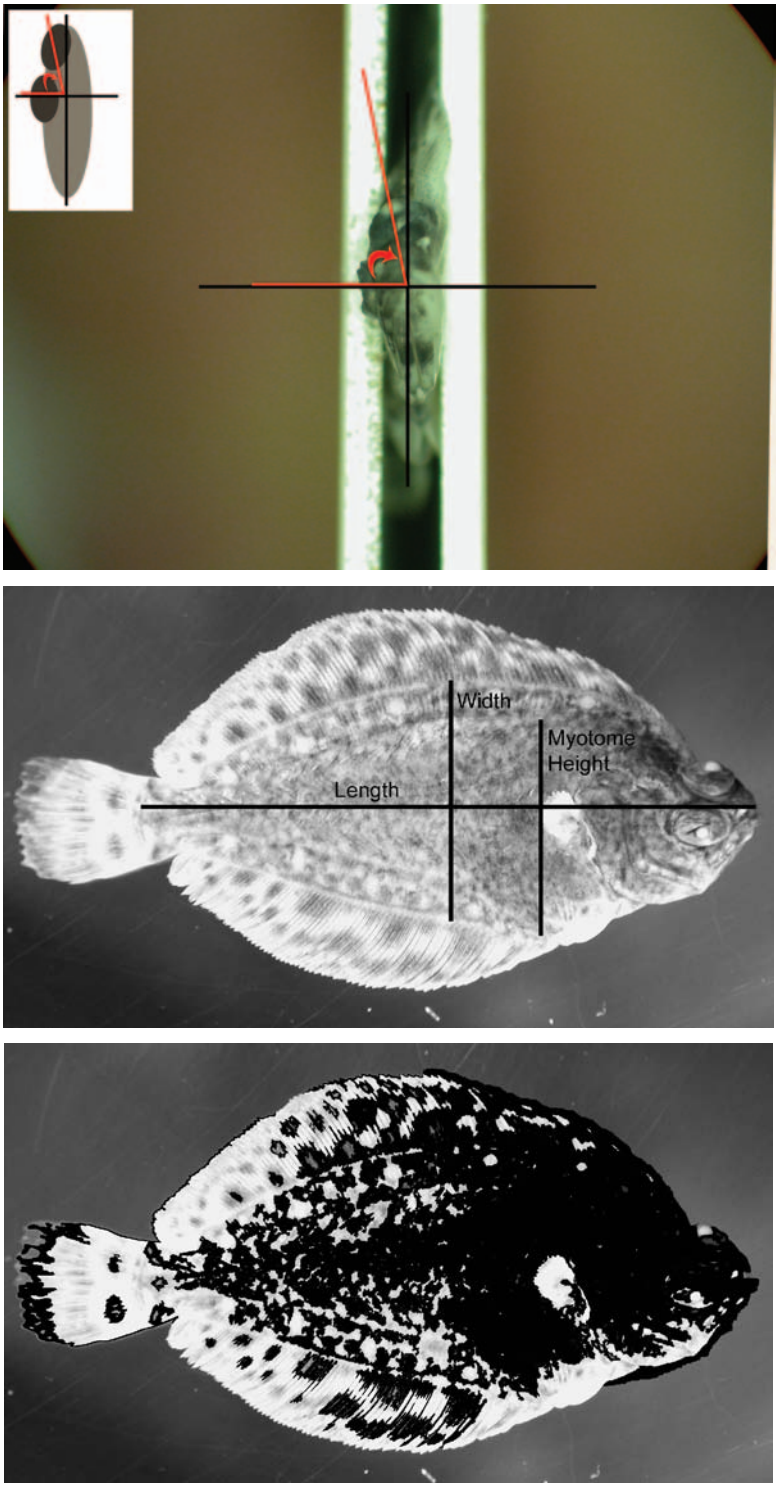
Pedigree analysis and genetic variation

We have previously shown that the 145 Atlantic halibut produced in 1996 and retained as potential broodstock display significantly decreased genetic variability.[3] Of the 82 crosses performed in 1996, only 36% are represented in the offspring retained as F1 broodstock. Fifty-three per cent of these F1 fish belong to four full-sib families, 89% to six maternal half-sib families, and 80% to five paternal half-sib families. Although heterozygosity values (Table 1) don't indicate a significant degree of inbreeding, the allelic variation of the F1 offspring decreased by 26% compared to the wild fish sample.

"Although heterozygosity values . . . don't indicate a significant degree of inbreeding, the allelic variation of the F1 offspring decreased by 26% compared to the wild fish sample."

Table 1. Genetic diversity of wild, parental and F1 halibut broodstock. The number of fish, the expected and observed heterozygosity (H_e and H_o), the number of alleles, the normalized number of alleles, and the number of alleles after exchange between the hatchery sites are shown.

Population	Number of fish	Expected heterozygosity	Observed heterozygosity	Number of alleles	Normalized number of alleles	Nunber of alleles after transfer of fish
Wild	52	0.841	0.838	16.0	13.35	
Parental	27	0.828	0.815	12.2	12.20	
1996 F1	145	0.795	0.815	11.8	9.90	
Site 1	43	0.776	0.809	9.8	9.30	12.20
Site 2	49	0.795	0.845	10.2	9.08	12.00
Site 3	53	0.772	0.792	10.8	9.48	11.80
Site 4 (1997 F1)	100	0.767	0.936	8.0	6.59	12.40



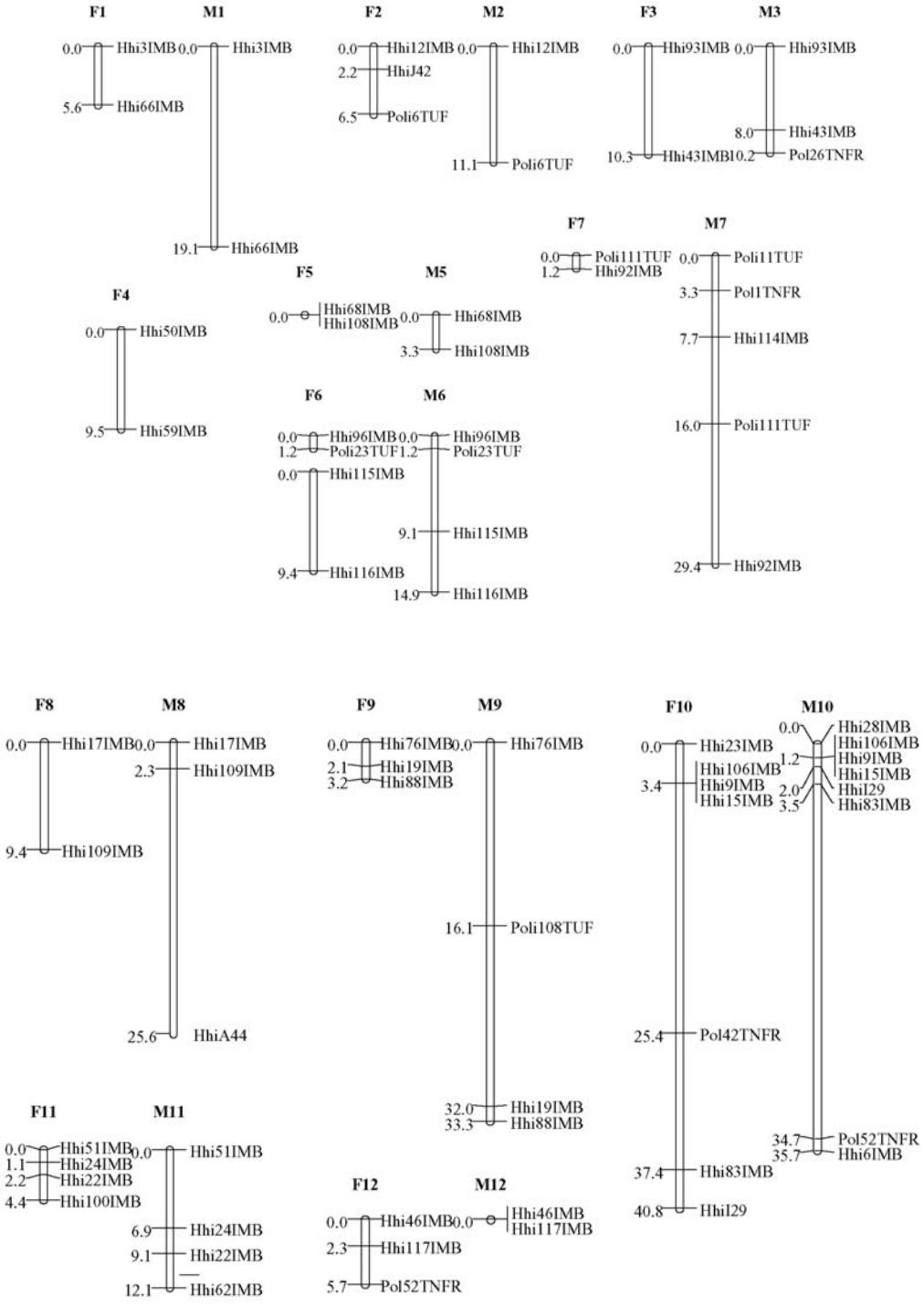
Given these decreases in genetic variability, transfers of the F1 broodstock between the hatcheries were one way to restore some of the lost variability. As well, an additional hatchery became involved as it faced similar problems because its F1 broodstock were the progeny of only 7 broodstock (2 females and 5 males). Pedigrees were also determined for the 1997 F1 broodstock from this hatchery. Based on the distribution of alleles and to minimize the number of fish moved, reciprocal transfers were designed that would help re-establish genetic variability. These transfers were carried out in March 2002. As can be seen in Table 1, allele numbers at all four sites have been returned to approximately the levels of the parental stock.

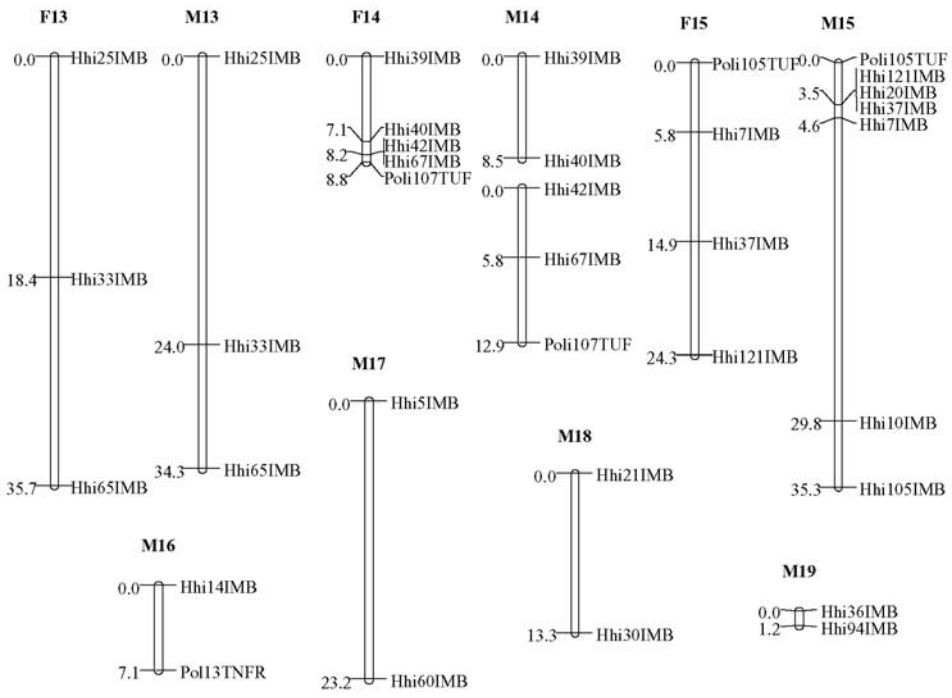
Morphometric analysis and genetic mapping

Three half-sib families were produced for genetic mapping and QTL analyses. Three hundred and twenty-three fish from these families were sampled just prior to weaning. The samples were sorted into families by genotyping all of them at a diagnostic microsatellite locus. Samples from two of the three families had statistically more robust distributions of the traits of interest and were selected for further analysis. Body size traits exhibited strong inter-correlation (weight to length, $r^2 = 0.93$; length to width, $r^2 = 0.91$). Eye migration, however, showed limited phenotypic correlation to body size ($r^2 = 0.47$ to 0.58) or pigmentation ($r^2 = 0.58$).

Figure 1. Morphometric data collected for a quantitative trait analysis in Atlantic halibut. All pictures are of the same fish. Top photograph: Head-on view of eye migration measurement in degrees. A properly migrated eye was around 70 whereas a non-migrated eye would remain near 180 (a diagramatic inset is provided for clarity). Middle photograph: Side-view used to measure fork-length, maximum width and myotome height. Bottom photograph: Approximation of the method used to evaluate % surface area pigmented (%SA); in this example %SA is ~60%. The true analysis using Simple PCI v4.0 Imaging Software (Compix Inc) is a comparative method based on a preset level of complete and non-existent pigmentation and the difference from each of these extremes is measured as a whole.

Figure 2. Current linkage map of Atlantic halibut based on 87 microsatellites (LOD of 3.0 ~ p < 0.001). A total of 19 unique sex-specific linkage groups are presented. Until accurate sex-specific recombination rates can be ascertained, both male (M) and female (F) linkage groups are shown. Distances are expressed in centiMorgans (cM), where 1cM = 1% recombination. Microsatellite naming follows that of Jackson et al.,[3] where Hhi is *Hippoglossus hippoglossus;* Pol is *Paralichthys olivaceus;* IMB is the National Research Council, Canada, Institute for Marine Biosciences; TNFR is the Tohoku National Fisheries Research Institute; and TUF is the Tokyo University of Fisheries.





A total of 87 microsatellites, 75 from Atlantic halibut and 12 from Japanese flounder, were used to genotype 92 progeny and both parents from each of the two mapping families. Sex-specific mapping identified 16 linkage groups in the female and 18 in the male at a LOD score greater than 3. In total, 19 unique linkage groups were identified (Fig. 2) with 18 and 9 markers remaining unlinked in the female and male respectively. Greater overall recombination was identified in male linkage groups (F:M = 0.92); however, this difference is not significant (G-test (1df) = 1.49; a significant G-test value is > 3.84).

Discussion

Pedigree analysis of offspring from one halibut production run using random mating showed that genetic variation can rapidly decrease if care is not taken to mitigate the problem early. Efforts should be made to maximize the effective population size (Ne) by careful selection of mated pairs and subsequent evaluation of offspring retained as broodstock. Until a focused selection program is initiated based on selection of preferred heritable traits, efforts should be made to maintain or increase genetic variation (i.e., number of alleles) in broodstock through transfer of fish between hatcheries or addition of wild fish.

This is the first report on the construction of a linkage map for Atlantic halibut. As more markers of various types are added to the map we expect significant changes in order and composition of the linkage arrangements presented here. Genetic markers are currently being added to the linkage map, including additional microsatellites from other flatfish. Amplified fragment length polymorphisms (AFLPs) will be added in the next year, and single nucleotide polymorphisms (SNPs) will be used to map genes and expressed sequence tags (ESTs) of interest.

To date only an exploratory QTL examination has been conducted. Preliminary results are promising, as several QTL affecting eye migration, pigmentation and

body size have been identified. However, as more markers are added to the map and more complete QTL analyses are conducted, the markers associated with the QTL are likely to change. These preliminary results suggest that strong QTL for these important traits will be identified.

Examination of recombination rates between common markers reveals a slightly higher recombination rate in the males than in the females. Male Atlantic halibut are the heterogametic sex and in many other organisms the heterogametic sex exhibits reduced recombination rates compared to the homogametic sex.[7] Although Coimbra et al.[4] also identified greater recombination in male Japanese flounder, their use of a gynogenetic male in the production of the mapping family confounds their results. A more robust analysis will be possible in Atlantic halibut as more markers are added to the linkage map.

Although the results are preliminary, they are encouraging from both a scientific and aquaculture perspective. Scientifically, the comparison between the halibut map and other flatfish will help examine functional and organizational evolution within the flatfish genome. The linkage map and associated QTL will allow for a more integrated breeding plan within the halibut industry which would include both phenotypic and genotypic selection of optimal performing broodstock.

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Oyster Breeding and the Use of Biotechnology

Ximing Guo

Oysters support major aquaculture industries worldwide. Despite the importance and long history of oyster culture, domestication and breeding of oysters are relatively new. Only recently have serious efforts been made toward the genetic improvement of oysters using traditional selective breeding and biotechnical approaches. One of the genetic technologies that have contributed to shellfish breeding is triploid-tetraploid technology. Triploid shellfish are useful for aquaculture because of their sterility, superior growth and improved meat quality. In almost all molluscan shellfish studied so far, triploids grow significantly faster than normal diploids, a phenomenon that has been referred to as triploid gigantism. The expression of triploid gigantism may be influenced by genetic and environmental factors. The sterility of triploids is not complete and may have important implications for the culture of both non-native and native species. Tetraploids are now available in three oyster species for triploid production. The application of molecular technology in oyster breeding is at an early stage, but holds considerable potential. Molecular markers have been developed and used for stock identification and inbreeding monitoring. Genetic linkage maps have been constructed and quantitative trait loci (QTL) are being mapped. The identification and mapping of economically important QTLs may greatly enhance our ability to improve oyster stocks for aquaculture.

"Almost all the oyster stocks used in aquaculture today remain largely wild and lack the desired characteristics for aquaculture."

Introduction

Oysters support major aquaculture industries worldwide. Aquaculture production of oysters amounted to 4.2 million metric tons in 2001, and its value, about US$3.5 billion, ranked fourth among aquaculture species after that of carps, shrimps and salmonids.[1] Oyster culture has a long history, which in China can be dated back over 2000 years. Despite the importance and long history of oyster culture, domestication and selective breeding in oysters are relatively new. To date, there are virtually no domesticated oyster species and few examples of well-documented breeding programs. Almost all the oyster stocks used in aquaculture today remain largely wild and lack the desired characteristics for aquaculture. Wild stocks often have slow growth and are not well adapted to hatchery and culture conditions. Only recently has the importance of domestication and genetic improvement been recognized and received attention. Progress has been made in selective breeding for disease resistance and growth using mass and family selections, and interstrain hybridization. Considerable research has been conducted on biotechnologies that can be applied to oyster breeding and there has been some success. This paper provides a brief review of oyster breeding, with an emphasis on biotechnical approaches.

Traditional Selective Breeding

Selection for disease resistance

Disease resistance is probably the most important trait for aquaculture species. The eastern oyster *(Crassostrea virginica)*, for example, faces three major diseases: MSX (caused by the parasite *Haplosporidium nelsoni)*, Dermo (caused by the parasite *Perkinsus marinus)* and juvenile oyster disease (JOD, possibly caused by a bacterium).[2] The first two diseases are the primary cause of the destruction of the oyster fisheries in the mid-Atlantic region of the United States, and they are also causing serious losses to the aquaculture industry. JOD has been a serious problem for oyster nurseries in the northeastern region of the US since 1988.

Selection for disease resistance is the main focus of an oyster breeding program based at the Haskin Shellfish Research Laboratory, Rutgers University. The program was established in the early 1960s by the late Dr. Harold Haskin and continues to this day. The initial goal of the selection program was resistance (or tolerance) to MSX. Strong resistance against MSX was achieved in multiple lines after five generations of mass selection.[3] Dermo disease appeared in Delaware Bay around 1990, and resistance to Dermo has been part of the selection program ever since. Moderate resistance to Dermo was obtained after about four generations of selection. Under Dermo exposure, the survival of the selected stock was about twice as high as that of the wild stock.[4] Similarly, four generations of selection at the Virginia Institute of Marine Sciences has led to significant improvement in Dermo resistance in the same species.[5]

In another effort in the eastern oyster, the Frank M. Flower Oyster Company (New York) produced oysters (FMF strain hereafter) that show greatly improved survival in the face of JOD, after just one generation of selection.[6] The FMF strain has been under long-term breeding in a commercial setting and is known for its fast growth. The FMF strain, however, has shown no or little resistance to MSX and Dermo. Hybrids between the Rutgers disease-resistant strain and the FMF strain survived as well as Rutgers strain (under Dermo) and grew as fast as the FMF strain.[4]

In the Sydney rock oyster *(Saccostrea glomerata)*, two generations of selection resulted in a 22% reduction in mortality from QX diseases caused by the parasite *Marteilia sydneyi*.[7] In the Pacific oyster *(C. gigas)*, three generations of selection reduced summer mortality by two-thirds.[8] Similarly, rapid response to selection and a high heritability (0.81) were reported for resistance to summer mortality in Pacific oysters cultured in France.[9]

Data so far seem to suggest that disease resistance in oysters has a strong genetic determination and can be efficiently selected for and even transferred through interstrain crosses.

"Data . . . suggest that disease resistance in oysters has a strong genetic determination and can be efficiently selected for and even transferred through interstrain crosses."

Selection for fast growth

Selection for fast growth has also been successful in oysters. In the eastern oyster, long-term selection by commercial growers improved the growth rate by 28%.[10] One generation of mass selection in the European flat oyster, *Ostrea edulis,* resulted in a growth increase of 21% to 42%.[11] In the Sydney rock oyster, two generations of selection yielded a gain of 18% in whole weight.[12] Recently, a large-scale and family-based selection program was established at Oregon State University targeting primarily fast growth in the Pacific oyster. An average gain of 9.5% in live weight was obtained after one generation of selection.[13] Heritability estimates for growth has been reported in several oyster species, typi-

cally ranging from 0.10 to 0.50.

The Triploid-Tetraploid Technology

At the present time, the triploid-tetraploid technology is probably the most important breeding technology for oysters. It has contributed significantly to oyster production. Triploids are organisms with three sets of chromosomes instead of the two that occur in normal diploids. Triploid molluscs are superior stocks for aquaculture because of their sterility, superior growth, improved meat quality and sometimes increased disease resistance. Triploid Pacific oysters are widely cultured on the west coast of the United States and account for about one-third of the total production.[14] They are also being cultured commercially in Australia, China, France and Chile. Commercial culture of triploid eastern oysters has begun recently in the US at pilot scales. Triploidy in the Pacific oyster and Suminoe oyster *(C. ariakensis)* have made field trials of the two non-native species possible on the Atlantic coast of the United States.

Growth of triploid oysters

". . . large body size is a general feature of triploid molluscs, a phenomenon . . . referred to as triploid gigantism."

Triploids are particularly powerful in growth enhancement of cultured molluscs. A review of available data suggests that superior growth is a general feature of triploid molluscs. Triploids have been studied in over 20 molluscs and significantly faster growth has been reported for triploids in almost all molluscs studied so far. Triploids grow faster than normal diploids by 30% to 44% in most species, and by 60% to 72% in some species.[15,16] Superior growth has been reported in all oyster species studied so far.

Several general observations can be made on the growth performance of triploid molluscs. First, superior growth or large body size is a general feature of triploid molluscs, a phenomenon that is referred to as triploid gigantism.[15] Triploid gigantism separates molluscs from finfish, where triploids are not generally larger than diploids. The inability of molluscs to regulate cell number is probably the fundamental cause of triploid gigantism. The large cells in triploids lead to large organ and body sizes. Sterility and increased heterozygosity may provide additional advantages to triploids. Second, several types of triploids can be produced, and not all triploids are alike. Triploids produced from tetraploids and polar body I (PB1) inhibition grow faster than triploids produced by inhibiting polar body II (PB2). The growth of PB2-triploids is highly variable among families, probably due to differences in deleterious recessive genes that become homozygous upon PB2 inhibition.[17] Thirdly, the growth of triploids varies among environments, often with triploids outperforming diploids in productive waters. In nutrient-limiting environments, the advantages of triploids may be absent or delayed.

Meat quality

Another benefit of triploid molluscs is improved meat quality during the spawning season. Oysters donate a large portion (30% to 60%) of their body to gonad production, and sexual maturation and reproduction often lead to a reduction in meat quality in normal diploids. For the half-shell oyster industry, animals with excessive gonadal material cannot be marketed. Because triploids are largely sterile and have greatly reduced gonad development, the meat quality of triploids is less affected by reproduction. In the Pacific oyster, triploid molluscs provided a high quality product that can be sold year round.[18]

On the other hand, the meat quality of triploid oysters may be affected by the ap-

pearance of brown spots in the gonad area.[19] The brown spots are apparently related to the abnormal gametogenesis in triploids and are restricted to certain environments.

Disease resistance

Whether triploids are more resistant to specific pathogens or summer mortalities are debatable. Triploid and diploid eastern oysters are equally susceptible to Dermo,[20] but triploids are less susceptible to MSX than diploids.[21] In the Sydney rock oyster, the mortality of triploids is half that of normal diploids infected by the parasite *Mikrocytos roughleyi*.[22] There are variable observations on the summer survival of triploid oysters from different environments. Overall, the response of triploids to diseases and stress is poorly understood at this time.

Sterility

Sterility can be used for biological containment of aquaculture stocks to reduce environmental impact. In many cases, sterility is a requirement for the introduction and culture of non-native species. For native species, sterility of the cultured stock that is often genetically selected can reduce genetic pollution by cultured stocks resulting from escape and interbreeding with wild populations.

Because the presence of three sets of chromosomes is problematic for meiosis, triploids are expected to be sterile. In oysters and most other molluscs, however, sterility is often incomplete. The lack of complete sterility is caused by the high fecundity and low haploid chromosome number (n=10) of oysters. The large number of gametes produced, and the low chromosome number, means there is a high probability that gametes with viable chromosome numbers will be produced by random segregation.

In the Pacific oyster, for example, triploids produce on average 1.1 million eggs per female.[23,24] Triploid female x triploid male crosses produce viable offspring in 0.0085% of the eggs fertilized. While most (of the survivors are triploids, some of them are diploids and hyper- diploids (2n+1, 2n+2). Theoretically, in every million eggs produced from a triploid x triploid mating, about one normal diploid and eight viable hyperdiploids are expected, in addition to the relatively large numbers of viable triploids. Two other factors may also increase the risk when using triploids for biological containment: 1) the reversion of triploid cells to diploids, and 2) the often unavoidable contamination of triploids with diploids in the hatchery. A few diploids in a population of triploids can greatly increase the number of survivors due to triploid x diploid mating [23,24] When large numbers of triploid oysters are used for aquaculture, they may lead to the establishment of triploid and diploid populations over time. Therefore, triploidy in oysters can greatly reduce the unwanted reproduction of cultured stocks, but cannot offer complete containment of non-native species.

Tetraploid oysters

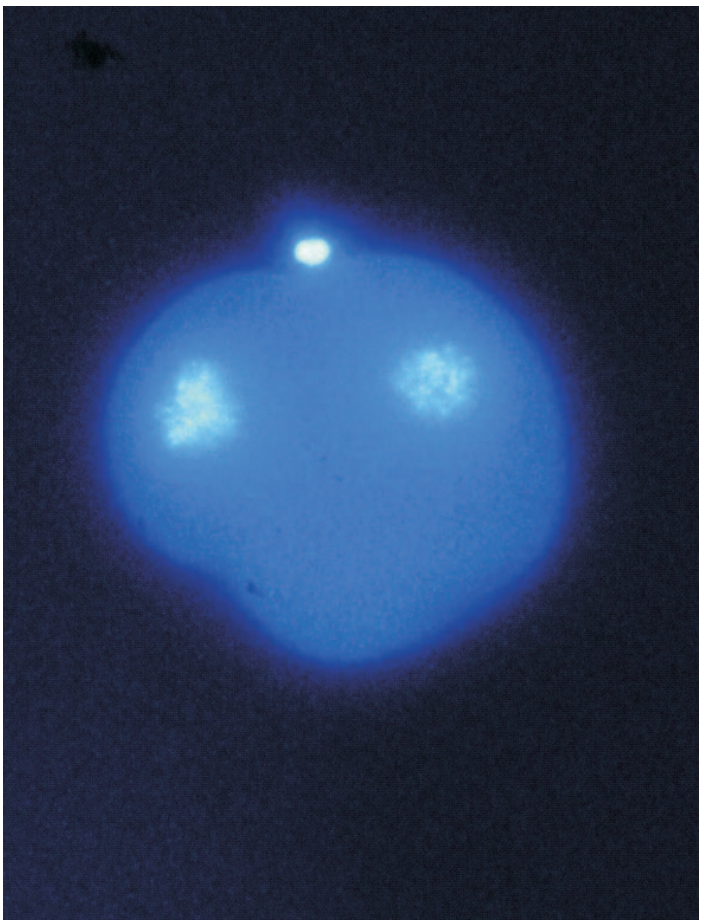
Traditionally, triploids are produced by blocking the release of PB2 with cytochalasin B (Fig. 1), a process that is highly technical, expensive and rarely 100% effective. Chemical treatment or blocking PB2 causes cellular damages and possible inbreeding, which affect survival and growth. The ideal way of producing triploids is through diploid x tetraploid mating. Tetraploids are organisms with four sets of chromosomes. They are fertile and produce triploids when mated with diploids.

Tetraploids have been successfully developed in three oysters so far: the Pacific oyster, the eastern oyster and the Suminoe oyster.[25-27] Tetraploids have revolutionized triploid production in oysters. Triploids produced from tetraploids are virtually 100% pure and grow faster than triploids produced from chemical induction (Fig. 2). Diploid x tetraploid crosses produced 99.7% triploids in the Pacific oyster and 98% in the eastern oyster.[28,29] Tetraploids are under development in several other molluscs including scallops, pearl oysters, clams and abalone.

Molecular Breeding Technology

Molecular biotechnology has tremendous potential for oyster breeding, although much of it has yet to be confirmed and realized. Genetic improvement of oysters ultimately depends on our abilities to understand and manipulate their genomes. Moderate progress has been made in some areas, including the use of molecular markers for pedigree analysis and genomic mapping.

Figure 1
Manipulation of polar bodies (PB) in oysters leads to the development of triploids and tetraploids: a Pacific oyster egg with only PB1 after inhibition of PB2.



Molecular markers

For a long time, allozyme markers were the only markers available for genetic analysis in oysters. The usefulness of allozyme markers is limited by the small number of loci available and the relatively low levels of polymorphism. In the past decade, several types of DNA markers have been developed in oysters, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and microsatellite (MS) markers.

The application of DNA markers has led to important advances in oyster genetics. The use of RFLPs helped to reveal population structures in the eastern oyster that were not known before.[30] The use of MS markers provided evidence of high genetic load in the Pacific oyster, which has important implications in oyster genetics and breeding.[31] MS markers have also been used for population and pedigree analysis.[32,33]

Genomics

The identification and mapping of economically important genes are essential for genetic improvement. The first step is to develop genetic markers and maps for the oyster genome. AFLP markers are particularly useful in genome mapping, because a large number of AFLP markers can be quickly generated for any mapping population without prior knowledge of DNA sequence. The use of automatic genetic analyzers makes fragment sizing and genotyping highly accurate and reproducible (Fig. 3). Although AFLPs are highly efficient, they are not as easily transferable as MS markers. MS markers are ideal for mapping analysis, but they are also expensive to develop and suffer from unusu-

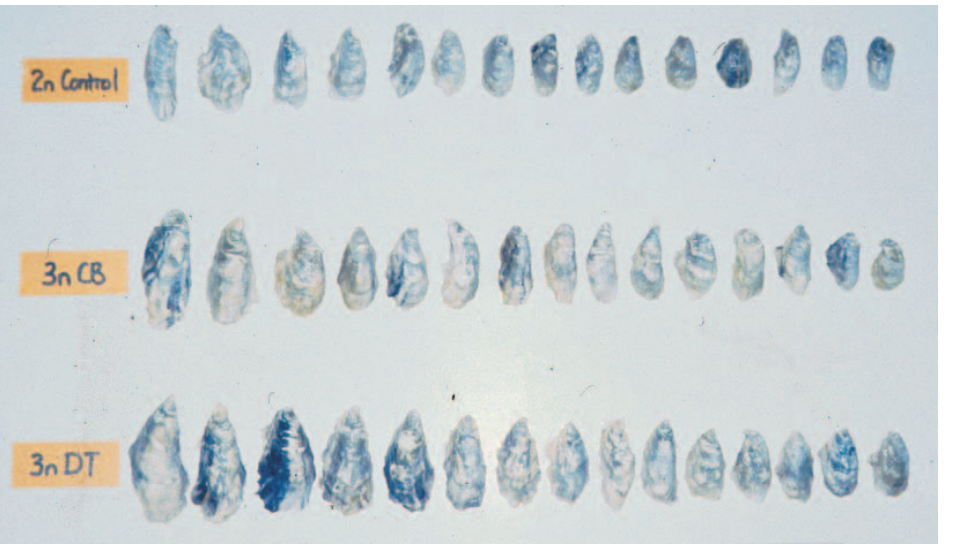


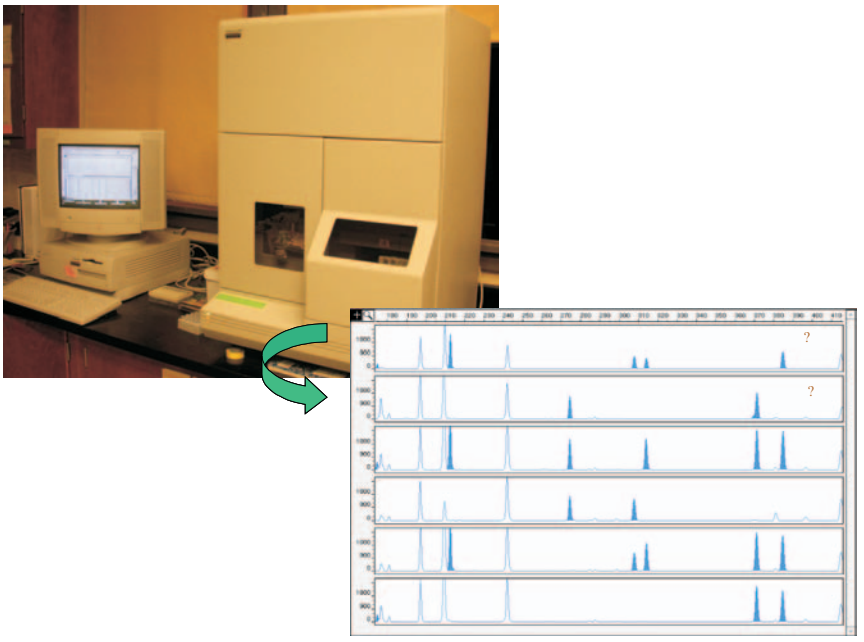
Figure 2
Not all triploids are alike: triploids produced from tetraploids (bottom) grow faster than triploids produced by blocking polar body II with CB (middle) and normal diploids (top).

ally high levels of null-alleles, segregation distortion and influence of repetitive elements in oysters.[31,34, 35] The best strategy for oysters is probably to use a limited number of MS markers to provide a transferable backbone and a large number of AFLP markers to saturate genetic maps.

Genetic linkage maps have been constructed for the eastern oyster using primarily AFLP markers plus a few MS and Type I markers.[36] The male map consisted of 114 markers in 12 linkage groups, covering 647 cM or 85% of the genome. Similarly, genetic linkage maps were constructed in the Pacific oyster using AFLP[37] and MS markers.[38] Disease-resistance QTLs are being mapped for the eastern oyster in our lab.[39] Other developments and trends in oyster genomics include: 1) the construction of a basic cytogenetic map in the eastern oyster by chromosomal mapping of P1 clones using fluorescence in situ hybridization;[40] 2) the large number of ESTs now available in GenBank; and 3) the active on-going development of Type I markers and mircro-arrays, and the proposal to sequence the oyster genome.[41]

The mapping of disease-resistance genes and other QTLs can lead to immediate application in marker-assisted selection (MAS). MAS is especially important for disease resistance in oysters, where sometimes breeding decisions have to be made when disease exposure is low or absent.

Figure 3
Automated genetic analyzer provides accurate and reproducible sizing and genotyping of AFLP markers.



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Robert Devlin

Production of All-Female Populations of Coho Salmon, *Oncorhynchus kisutch,* using Y-Chromosomal DNA Markers

Justin Henry, Dionne Sakhrani, and Robert H. Devlin

Enhancement of production of salmonids in aquaculture requires effective control of reproduction and growth, both of which have been the subject of significant biotechnological research. For Pacific salmon, monosex female chinook salmon strains have been utilized for many years to eliminate losses arising from precocious maturation of males. Y-chromosomal DNA markers can facilitate development and maintenance of monosex strains by allowing regular XY males to be reliably distinguished from masculinized XX males. Two markers are available for chinook salmon, OTY1 and GH-P, whereas only the latter marker reliably distinguishes genetic sex in coho salmon. Recently, the Department of Fisheries and Oceans and the aquaculture industry have worked together to develop monosex coho salmon, both to reduce precocious maturation and to enhance roe production.

Introduction

"Salmonids possess an XY genetic sex determination system which is highly stable, allowing production of pure populations of XX individuals for production purposes."

Control of reproduction has played an important role in development of salmonid aquaculture globally.[1] For many salmonids, one sex grows faster than the other or possesses maturation characteristics that are less desirable for production purposes. For example, males of coho *(Oncorhynchus kisutch)* and chinook salmon *(O. tshawytscha)* can undergo a precocious or early sexual maturation at a size that is less than maximally profitable. To alleviate this problem, all-female strains of chinook salmon were developed in the mid 1980s by Ed Donaldson and colleagues for application in the British Columbia aquaculture industry. Since that time, monosex strains have supplied the bulk of production of this species in Canada. Monosex strains have also been developed for other salmon species, including Atlantic salmon *(Salmo salar)* and rainbow trout *(O. mykiss)*.[2,3]

Salmonids possess an XY genetic sex determination system[4] which is highly stable, allowing production of pure populations of XX individuals for production purposes. Historically, this has been achieved by coupling sex reversal protocols with family selection. Mixed sex progeny are treated with androgen at the alevin stage which masculinizes XX females into functional males, yielding groups containing both XX and XY males.[5] The two types of males have been distinguished by 1) test crossing each individual with regular females, and retaining those which yield only female progeny,[6] 2) using distinguishing characteristics (i.e. lack of a sperm duct) that exist between XX and XY males in some but not all salmon species,[2,3] 3) identification of hermaphrodite gonads, which is strongly indicative of an XX genetic background, and 4) the use of Y-chromosomal DNA markers which can reliably distinguish males of XX and XY genotype.[7-9]

One salmonid species of commercial importance for which monosex strains

have not been developed is coho salmon. This species, while not providing the bulk of aquaculture production in North America, is an important production species globally, in particular with high levels of production in Chile. Coho salmon do show moderate to high levels of precocious maturation in some strains, and thus the development of all-female strains would be of significant benefit for aquaculture. Here we describe the development of all-female strains of coho salmon using a Y-chromosomal DNA marker in close proximity to the sex determination locus[9] which provides reliable ability to identify XX males in mixed genetic sex populations derived from androgen treatment.

Materials and Methods

Coho salmon were derived from aquaculture broodstock maintained at Target Marine Hatcheries, Sechelt, BC. Androgen treatments were performed essentially as described[10,11] using methyltestosterone and methyldihydrotestosterone as the masculinizing agents. Mixed sex alevin (both XY and XX) derived from regular crosses were utilized for androgen treatments. Fish were reared in fresh water until smolting, at which time they were transferred to sea pens for growth until sexual maturity. Broodstock were transferred to a freshwater site, individually tagged, and blood samples taken for genotyping as described below. Following determination of genetic sex, genetic females (XX) were selected (based on molecular testing; see below) and examined for evidence of masculinization (milt production and/or morphological characteristics). If produced, sperm were collected directly from sperm ducts. All other genetic females were euthanized and their gonadal tissue examined for signs of testicular development in which case sperm were collected by gentle maceration of excised testicular tissue and collection of milt. Milt was used in crosses with regular female (XX) broodstock and progeny reared in fresh water until presmolt stages. Progeny were sampled and also subjected to sex marker genotyping to verify their monosex condition as described below.

"Coho salmon do show moderate to high levels of precocious maturation in some strains, and thus the development of all-female strains would be of significant benefit for aquaculture."

Blood was sampled from adult fish by puncturing the haemal arch with an 18-gauge needle (without syringe), allowing blood to fill the needle. Two L of blood were collected from the socket of the needle. These blood samples were immediately injected into 100 L of 0.01 N NaOH and the mixture re-pipetted several times to ensure a homogeneous mixture. Samples were then shipped to the laboratory in West Vancouver.

Determination of genetic sex (XX vs XY) was performed using a genotyping test designed to detect the coho salmon Y chromosome.[12] This assay is based on the presence of a growth hormone pseudogene (GH-P) on the Y chromosome which can be distinguished from other autosomal copies of GH genes using simple PCR-based diagnostics. The PCR test utilized primers GH5 and GH6[13] which span intron E of all GH genes in the genome. Polymerase chain reactions were performed using annealing temperatures between 50 C and 60 C in reaction conditions as described,[14] and products were electrophoresed in 0.8% agarose gels stained with ethidium bromide. Oligonucleotide sequences used as amplification primers are: GH5: 5 -AGCCTGGATGACAATGACTC-3 and
G CTACAGAGTGCAGTTGGCCT

Results

The Y-chromosomal marker (GH-P) previously found to be closely linked to the sex determination locus in wild coho salmon was also found to be Y-linked in the

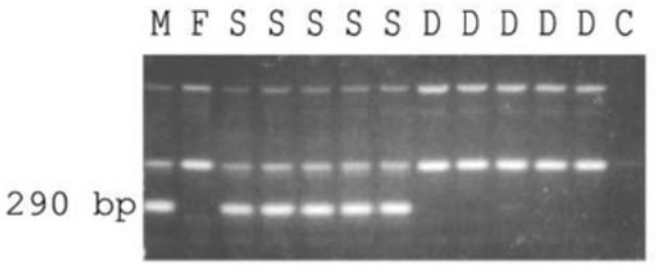


Figure 1
Agarose gel showing sex-specific PCR amplification of the Y-linked GH-P fragment (290 bp) in coho salmon. M, mother; F, father; S, sons; D, daughters; C, no template control.

domesticated population (unpublished data). Thus, sex genotype can be readily determined by application of a simple PCR diagnostic which can be performed using crude tissue or blood preparations. The GH primers used for amplification yield two products in genetic females (derived from GH1 and GH2), whereas in males these fragments plus a band derived from the Y-linked GH pseudogene are produced (Fig. 1). The assays can be rapidly conducted such that under ideal conditions, data can be generated and returned to the broodstock site within a single day.

Over a 5-year period, coho salmon were masculinized with methyltestosterone and reared to sexual maturity for use as brood stock. At maturation, blood was drawn and genotyped for genetic sex (Table 1). Phenotypic males of female (XX) genotype were selected for use as broodstock and used in crosses with regular females. In the first year of production, a high proportion of females were obtained in progeny derived from such crosses (Table 1). Exceptional males were recovered in the second year, and these were tested and found to contain a Y chromosome. Thus, exceptional males did not appear to be arising from autosomal genetic effects or environmental influences, but rather most likely arose by the accidental inclusion of XY brood males into production crosses. In subsequent years, 100% female progeny have been observed, demonstrating the utility of this approach in the production of monosex populations for this species. The overall procedure used for sex reversal is shown in Figure 2.

In addition to benefits of monosex strains derived from elimination of sexual maturity, all-female strains also allow for enhanced roe production which can be of benefit if appropriate markets have been developed. Thus, for coho salmon, the use of monosex female strains has allowed a doubling in roe production from the same number of production animals previously used in mixed sex culture (Fig. 3).

Discussion

Monosex strains of many species of fish have been developed for use in aquaculture.[4] For species such as tilapias, production of all-male progeny is desirable,[15] whereas for other species females are the preferred sex. For species which have polygenic or environmental sex determination systems, the development of stable monosex strains can be problematic due to the rare but regular occurrence of individuals of the non-desired sex. While this is not a direct problem for production purposes, if such exceptional fish are sex reversed and used as broodstock, larger numbers of exceptional individuals will result in subsequent production generations. Thus, utilizing genetic markers which appear to be genetically inseparable from the sex-determination locus, and can thus reliably determine the genetic sex of individuals, is of significant benefit for development of stable monosex populations of fish for pro-

Table 1. Production of broodstock and monosex progeny.

Brood year	1999	2000	2001	2002	2003
Brood tested	522	591	582	158	210
% Masculinized	1	4	33	41	86
% Nonmaturing	9	38	55	41	14
Number of progeny smolts tested	1600	1600	1600	600	600
Progeny — % female	100	95	100	100	100

duction. Variable microsatellite markers can also be used in monosex strain development if they are closely linked to the Sex locus, and the linkage phase of alleles at the locus and the sex determination locus are known in all broodstock.

Some data are available comparing the growth of mixed sex and monosex populations under production conditions. An economic analysis of these differences for chinook salmon indicate a significant advantage,[16] and analysis of growth and feed conversion efficiency also indicate an advantage for monosex catfish culture.[17] In the case of tilapias, monosex male culture is advantageous due to the elimination (or strong reduction) of breeding in pond culture which can result in high densities and consequent reduced fish size and overall production value.[15,18] In the case of tilapias, YY strains have been developed[19] which allow, when crossed to regular females, production populations largely consisting of XY males (some polygenic autosomal influences on sex determination exist in tilapia species). For salmonids, all-male populations derived from YY fathers have been produced for rainbow trout[20,21] and chinook salmon,[9] but these strains have not seen wide use commercially at this time.

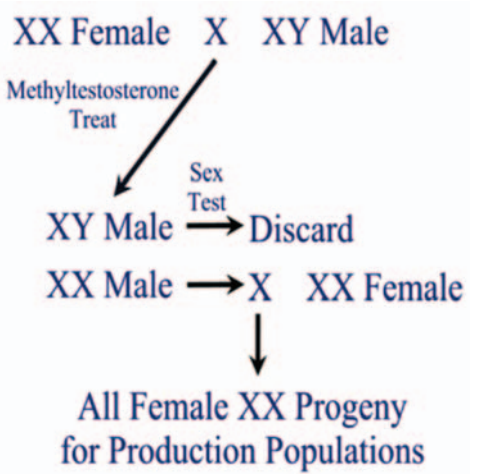
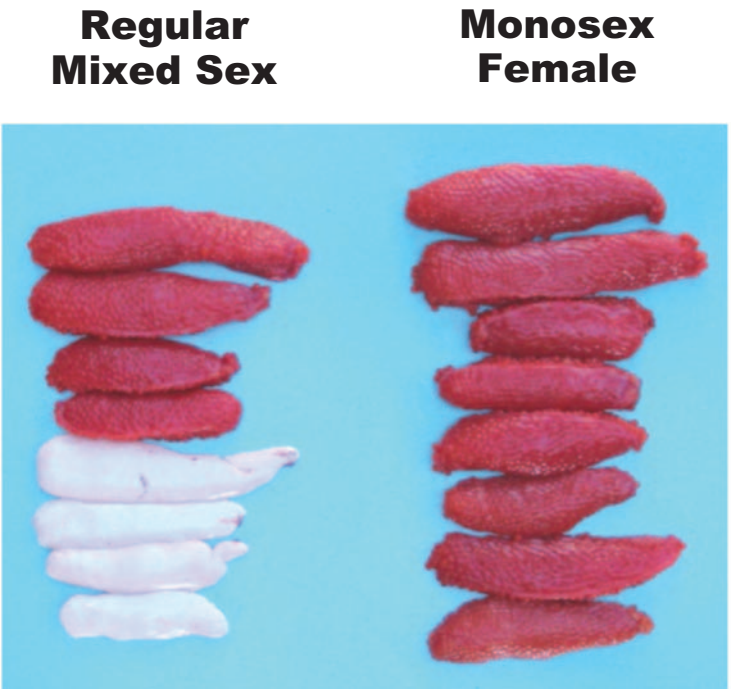


Figure 2
Protocol for development of all-female progeny using Y markers.

While the primary purpose of monosex strain development for aquaculture is to enhance production, in some cases single sex populations may provide an environmental benefit as well. For example, in areas where non-native species are being cultured, the use of single sex populations, if adopted universally, can provide a highly effective method for reproductive containment.[22] Such fish may live out their life following escape, but with no conspecifics in nature of the opposite sex with which to breed, their direct impact would be limited to a single generation and would be expected to be of limited magnitude. Monosex strains also have utility in the production of nonreproductive populations of triploid fish. For many species, triploid males still undergo sexual maturation and produce functional although aneuploid sperm, whereas females do not show significant ovarian development.[23] Thus, all-female triploid populations are advantageous for purposes of reproductive containment and for suppression of sexual maturation.

Figure 3
Enhanced production of roe from all-female strains.



Acknowledgements

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Production of All-Female and Sterile Populations of Fish for Aquaculture

Tillmann Benfey

There are a number of reasons to consider sex control as a practical application in commercial fish culture. For instance, monosex populations are of use in species where one sex exhibits superior culture characteristics compared to the other, whereas sterile populations can be used to prevent early maturation, to protect investments made in developing superior lines of fish or to address concerns about environmental impacts of aquaculture escapes. Sex control is achieved by manipulating inheritance at the time of fertilization or by altering subsequent gonadal differentiation through the manipulation of rearing temperature or the administration of steroids at key developmental stages. All-female populations of lumpfish, Atlantic halibut and shortnose sturgeon have been produced for pilot-scale evaluation in Atlantic Canada but have yet to be used commercially. Triploid (sterile) Atlantic salmon have been evaluated through commercial production in New Brunswick and elsewhere, with limited success to date.

Fish are unique among farmed vertebrates in the ease with which single-sex or sterile populations can be mass produced through simple, inexpensive manipulations. This paper provides a brief overview of the rationale and methods employed for such manipulations for fish culture, with an emphasis on coldwater species farmed in Atlantic Canada.

"Fish are unique among farmed vertebrates in the ease with which single-sex or sterile populations can be mass produced through simple, inexpensive manipulations."

Production of Single-Sex Populations

Although it is possible to produce both all-male and all-female populations of fish, the latter approach is of specific relevance to coldwater aquaculture in Atlantic Canada. Many, if not all, of the species of fish currently farmed commercially or on a pilot-scale in Atlantic Canada have biological characteristics making females more valuable than males as production animals. In flatfish such as the halibut, for instance, juvenile females grow faster than juvenile males. Female flatfish, eels and many salmonids also mature later in life than males and therefore reach larger ultimate sizes. And in the case of sturgeon and lumpfish, it is the females that produce the roe which make these species of interest for farming. All-female populations of fish can readily be produced by genetic and endocrine manipulations, and possibly also through temperature manipulations.

Genetic Manipulation of Sex Ratio

Uniparental maternal or paternal inheritance—respectively referred to as gynogenesis and androgenesis—can be used to alter sex ratios in fish populations. In species having female homogamety (equivalent to the mammalian

XX-female, XY-male system), exclusion of the paternal genome through gynogenesis results in populations comprised entirely of females. This is well established for salmonid fishes[1] and has also recently been demonstrated for Atlantic halibut.[2] Alternatively, in species with male homogamety (equivalent to the avian WZ-female, ZZ-male system), gynogenesis yields populations comprised mostly of normal males and "super-females" (i.e., WW genotype), with the possibility of smaller number of normal females. Sex ratios recently obtained from gynogenetic populations of shortnose sturgeon suggest that this species has male homogamety.[3] The creation of all-female populations in such cases requires the mating of normal males with super-females. Exclusion of the maternal genome through androgenesis yields populations comprised of normal females and "super-males" (YY genotype) in species with female homogamety, and in all-male populations in species with male homogamety.

The production of gynogenetic and androgenetic populations requires the use of radiation to destroy or otherwise inactivate the genome that is to be eliminated, as well as simple thermal or hydrostatic pressure treatments to duplicate the genome that is to be retained. Thus, gynogenesis is typically a 2-step process of first exposing sperm to ultraviolet radiation at doses sufficient to affect conformational changes in genomic DNA structure—thereby preventing its duplication—followed by thermal or pressure treatments applied to eggs shortly after activation with UV-irradiated sperm (Fig. 1). Depending upon their timing, these thermal/pressure treatments duplicate the maternal genome either through retention of the second polar body or by blocking the first cell division. The second polar body is a product of the meiotic reduction division in the egg, a process triggered by the sperm cell penetrating the egg. Gynogenetic fish produced in this way have reduced heterozygosity but are not completely homozygous. Alternatively, if meiosis is allowed to go to completion but the first mitotic division is blocked, the resulting fish will be completely homozygous and can be used to develop clonal populations.

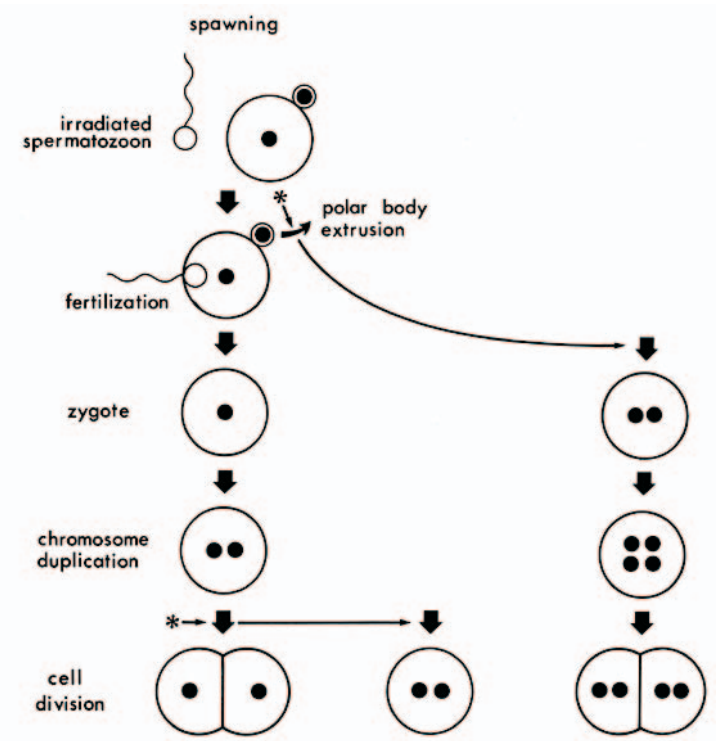

Figure 1
Schematic representation of gynogenesis induction. Black circles represent haploid maternal chromosome sets originating from the egg and asterisks denote thermal/pressure treatments. Activation of development with irradiated sperm leads to the production of haploid embryos (left column). If this is followed by treatments to block the first mitotic division (middle column) or extrusion of the second polar body (right column), viable diploid embryos are produced.

Endocrine Manipulation of Sex Ratio

Although sex is ultimately under genotypic control and is often determined—and fixed—at fertilization, the phenotypic expression of sex (i.e., gonadal differentiation into ovaries or testes) is mediated by sex steroids. Thus, estrogens and androgens have feminizing and masculinizing effects, respectively. These hormones are the natural "sex inducers" in vertebrates, and their exogenous administration during gonadal differentiation can be used to change sex in fish.[4] In some species this developmental process occurs prior to yolk absorption, requiring the immersion of embryos in steroid solutions. In species where gonadal differentiation occurs later in

life, these steroids can be administered through the food, either via live prey (e.g., lumpfish[5]) or in prepared feeds (e.g., Atlantic halibut[6] and shortnose sturgeon[7]).

Although it is possible to achieve "direct" feminization of fish populations by the administration of estrogens, the marketability of such fish for human consumption is affected by their exposure to steroids. The preferred method for aquaculture applications is therefore "indirect" feminization. For instance, the appropriate administration of androgens to salmonid fishes results in all-male populations.[4] If the sex-reversed genotypic females ("neo-males") within such populations are subsequently mated to normal females, all the resulting offspring will be female but will not have themselves been exposed to steroids. This method for the production of all-female populations for aquaculture is currently used for salmonid species such as rainbow trout and chinook salmon,[4] and should also be effective for Atlantic halibut.[6]

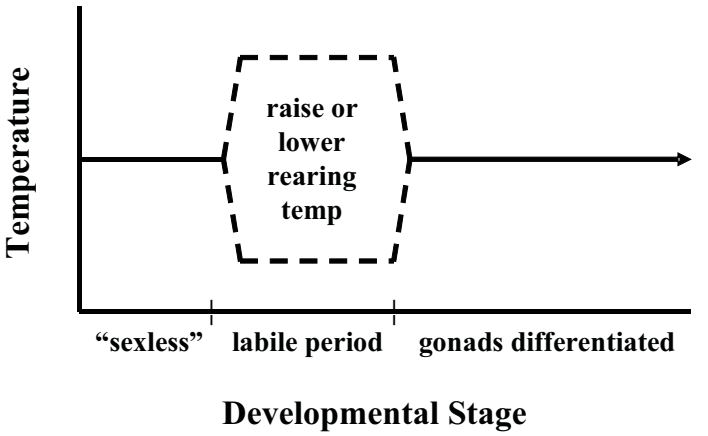

Figure 2
Schematic representation of temperature-induced sex reversal. The developmental time-line begins with fertilization, at which time genotypic sex is determined but the animal is phenotypically sexless. At some stage during development the differentiating gonads are sensitive to environmental manipulations; this is referred to as the "labile period". As development continues, gonadal differentiation progresses to a stage after which environmental manipulations are no longer effective.

Environmental Manipulation of Sex Ratio

All steroid hormones are derived from cholesterol, with their biosynthesis controlled by specific steroidogenic enzymes. Chemical inhibition of such enzymes has been used to create single-sex populations of fish.[8] Given that most enzymes are highly sensitive to environmental conditions, it should also be possible to manipulate sex ratios in fish by manipulating the expression and/or activity of steroidogenic enzymes.[9] The best candidate for such environmental manipulations is temperature, given the well documented effects of egg incubation temperature on sex ratios in reptiles and some species of

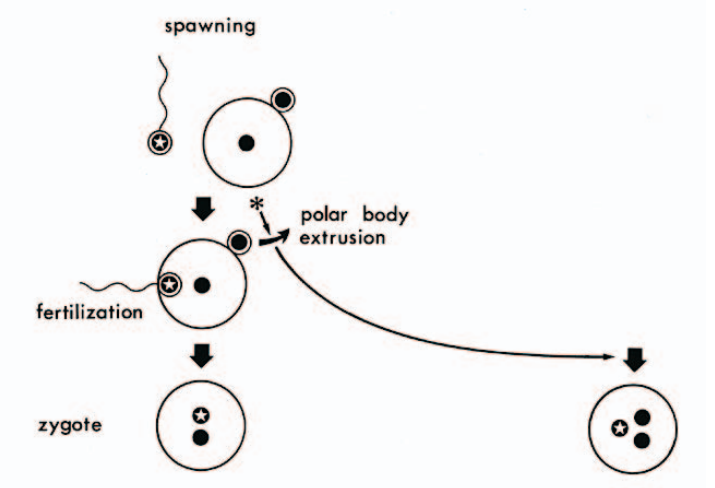

Figure 3
Schematic representation of triploidy induction. Black circles and white stars represent haploid maternal and paternal chromosome sets originating from the egg and sperm, respectively, and the asterisk denotes a thermal/pressure treatment. Normal fertilization leads to the production of diploid embryos (left column), but if this is followed by treatments to block extrusion of the second polar body (right column), viable triploid embryos are produced.

fish. Such a protocol (Fig. 2) has obvious attractiveness for aquaculture because it represents a more consumer-friendly approach, given the absence of genetic manipulation or use of steroid hormones. However, recent experiments with Atlantic halibut, where fish were reared at either high or low temperatures through the previously defined period of gonadal differentiation, failed to effect a change in sex ratios.[10]

Production of Sterile Populations

There are three basic applications for sterile populations of fish in aquaculture. Firstly, it eliminates production losses due to early (pre-harvest) sexual maturation. Such losses can be due to reduced growth rate and flesh quality, changes in external appearance and/or increased mortality. Secondly, sterility reduces the risk of environmental impacts from the escape or intentional release of genetically modified or non-native (exotic) fish. Genetic modifications can range from simple domestication through transgenesis. Although sterility does not address most of the possible immediate environmental impacts of such fish, it does ensure that these impacts cannot be sustained through feral spawning. And lastly, sterility can be used to protect investments made by breeders in the development of unique genotypes developed through selective breeding or advanced biotechnology.

Figure 4
Characteristic lower jaw deformity in a market-size triploid Atlantic salmon.

Although there are a number of ways by which sterile populations of fish can be produced,[11,12] induced triploidy is currently the only method available for commercial aquaculture. The methods used for producing triploid populations of fish are similar to those used for production of gynogenetic populations, but without any special treatment of the sperm (Fig. 3). The culture characteristics of triploid Atlantic salmon have been investigated in some detail. Although they generally have good freshwater growth, survival and smolting rates, their marine survival is reduced and—at least in some populations—there can be a high incidence of a characteristic lower jaw deformity.[13-15] This deformity (Fig. 4) does not affect body conformation or growth rates, but does require that fish be sold head-off or be further processed (fillets, steaks, etc.) before sale. The oft-observed reduced performance of triploid salmonids in commercial culture may reflect a reduced ability to withstand chronic stress.[15] That triploids have different culture characteristics from diploids likely reflects limitations of triploids imposed by their genetics and physiology.[16] It is important for farmers to appreciate that triploids, aside from being sterile, are biologically quite different from conspecific diploids, and that culture conditions may need to be modified for triploids to thrive.

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Intellectual Property and Intellectual Wealth

Keith Culver

Almost any discussion of aquaculture biotechnology research soon runs into talk about intellectual property (IP). As funding bodies, researchers, and end-users of research talk about IP, a bewildering range of other terms are brought into the mix, from 'discovery' through 'innovation' and on to 'commercialization' and 'transfer' of knowledge, technology, and material. These terms are apparently connected to 'competitiveness', and most recently and more tentatively, 'knowledge management'. Do we all mean the same thing as we use these terms? How important is possession of IP to achievement of commercialization, knowledge transfer, and competitiveness? This discussion identifies and bridges the divide between funders', researchers', and end-users' talk of IP in research and development. Knowledge management programs are identified as a common ground for reconciliation of these groups' sometimes divergent interests. A knowledge management program allows all partners in R&D to use IP rights to best advantage, recognising that knowledge transfer, commercialisation and competitive advantage can often be secured by means which do not involve the effort and expense associated with IP right assertion and defence.

". . . biotechnology is increasingly seen as a means to national wealth."

No discussion of aquaculture biotechnology R&D gets very far without talk of intellectual property (IP). Everyone has something to say about IP. Some want to create it, some want to use it, and some want to count it. Others wonder whether it has any place in aquaculture biotechnology. Who holds these diverse views, and why? Are different views evidence of healthy disagreement, or more dangerously, a fundamental obstacle to collaboration between government, research institutions, researchers and the private sector? This discussion explores these views and describes the beginning of a resolution to some of the disagreements over the place of intellectual property in the research-to-solution cycle of aquaculture biotechnology. Collaborative R&D is most likely to succeed when IP is regarded as just one element in each partner's contribution of measurable, reportable 'intellectual wealth.'

Government, Applied Biotechnology, Intellectual Property and the Power of Prosperity

How does government view applied biotechnology? In a word, ambivalently. While government retains its responsibility to regulate applied science for the public good, biotechnology is increasingly seen as a means to national wealth. This emphasis is made clear in the recently released Canadian Biotechnology Strategy document *Biotechnology Transforming Society: Creating an Innovative Economy and a Higher Quality of Life*.[(1)]

Prosperity is explicitly linked to research:

> "Prosperity is measured globally in today's economy in the currency of knowledge. Countries that succeed recognize that the greatest benefits derive from a readiness to innovate, to accept change, to embrace new ideas, to take greater risks. If Canada is to secure a competitive advantage in the global, knowledge-based economy, our country must proactively pursue research discoveries that will lead to innovative products and services . . ." (XII Research Funding Agencies, para 1.)[2]

Research is explicitly linked to biotechnology discoveries:

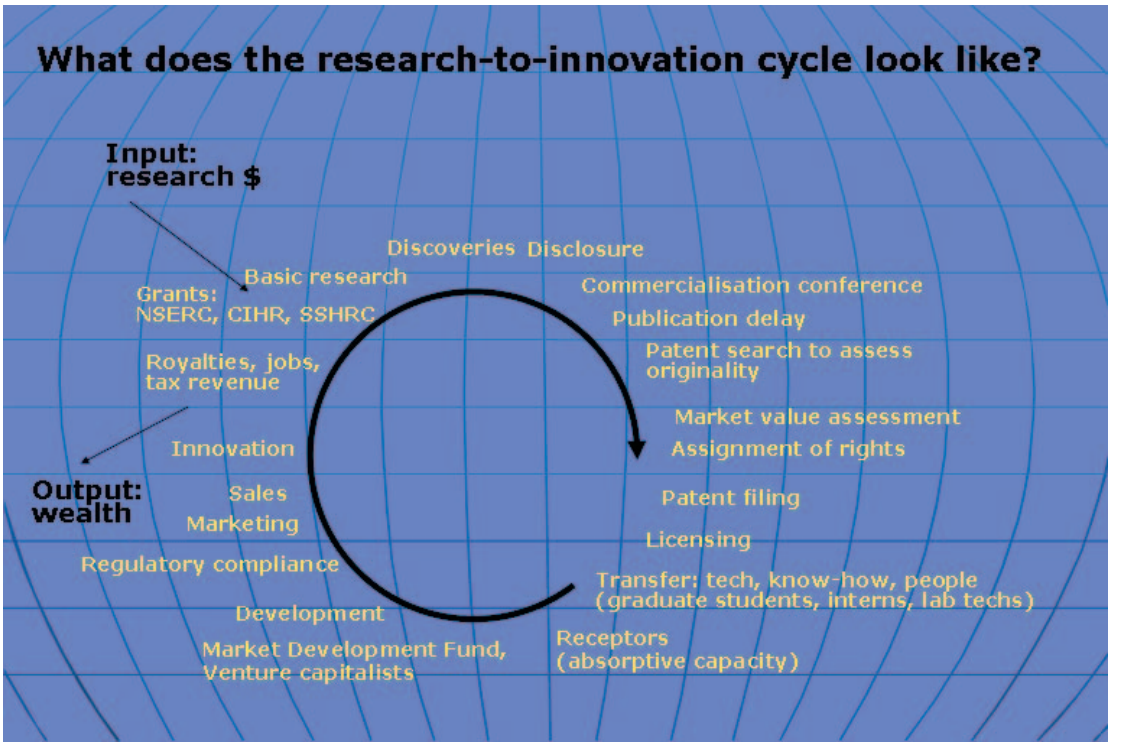
> "An explosion of extraordinary advances in molecular biology, genetics and biochemistry—the life sciences—has spawned a broad array of biotechnology products and services which are transforming everything from the foods people eat, to the integrity of the physical environment, to the quality of health care Canadians receive. A key contributor to the knowledge-based economy, biotechnology generates new jobs and business opportunities, and supports the competitiveness of some of Canada's most important industries." (IX Industry Canada, para 1.) [3]

Biotechnology discoveries are explicitly linked to intellectual property protection:

> "The growth of the biotechnology industry requires world-class business and regulatory regimes that nurture innovation and build public trust and confidence. Industry Canada supports such a competitive business climate by monitoring key marketplace frameworks such as intellectual property and regulations."[4]

For government, prosperity is measured in the currency of knowledge. One unit of that currency is intellectual property rights in biotechnology products and services, generated from research funded by "proactive" investment using existing funds. Government views applied biotechnology as part of a **manageable cycle** of wealth-creation. The cycle runs from aquaculture biotechnology research to innovation and marketplace success, circulating back to reinvestment of national wealth in further research.There is no single, agreed picture of the cycle (Fig. 1), yet its core elements are beyond dispute in the government view of the research-to-innovation cycle.

Intellectual property rights serve three purposes in government's attempts to manage the research-to-innovation cycle in aquaculture biotechnology. First, intellectual property rights serve government's interest in **economic development** by providing an incentive to private sector firms to use biotechnology research results. Intellectual property rights provide an easily transferred, shareable, yet exclusive right to creation of a product or process. Right-holders gain a means of defence against theft of their investment in development of biotechnology discoveries into marketable, innovative products and processes. Intellectual property rights can also serve as instruments for government intervention in the marketplace: government can retain a share in intellectual property rights generated by nationally-subsidised research, and use its partial ownership to influence how those rights are used. Government sometimes functions as a 'knowledge broker,' working to give Canadian firms access to intellectual property needed to gain a competitive advantage in the global marketplace.



Explanatory Legend

- **Basic research**, sometimes called pure or fundamental research, is conducted using funds identified as **grants**, gained from **NSERC, CIHR**, and **SSHRC**.
- Research results or **discoveries** (**NSERC** now issues 'discovery grants' instead of standard grants) are potential **innovations**—wholly new products or processes whose merits allow them to displace formerly dominant products or processes.
- Researchers **disclose** research results that can be commericalized and **commercialisation** conferences bring scientists, universities and funders to examine a **market value assessment** for a particular research result.
- Scientists sign over control of their discovery to commercialisers in return for a **share** in the **intellectual property rights** and r**oyalties** from **license** use of that **IP transferred** or to someone with an interest in it and the ability to pay for it.
- **Receptors** of research results have varying **absorptive capacity** or readiness to work with **venture capitalists** or **development funds** to turn a research result into a product or process which complies with legal **regulations** and can be **marketed** and **sold** as a market-dominating **innovation**.
- Canada benefits as the nation retains a share of **royalties**, or Canadian firms which commercialise innovations are able to **compete successfully in world markets**, generating **jobs** and **personal wealth** for Canadians who pay **taxes** and enable government to provide social services and national wealth for re-investment into basic research.

Second, intellectual property rights serve as an easily counted, common denominator expression of scientific discoveries. Intellectual property is in this way both an **accounting and accountability tool**. Note, for example, that the very first sentence of NSERC's account of its mission is framed in terms of return on investment: "NSERC's role is to make investments in people, discovery, and innovation for the benefit of all Canadians."[5]

Third, government can use information about successful producers of intellectual property in its attempts to **control the cycle.** Genome Canada, for example,

asks investigators to list patents and copyrights held and emerging from the research. Similarly, progress reviews in Networks of Centres of Excellence require investigators to identify intellectual property emerging from their research together with steps taken to safeguard that intellectual property. Intellectual property is not just an output of the research conducted under these frameworks: it is a crucial success indicator and plays an increasingly important role in governments setting the incentives to which research scientists respond. Genome Canada and Network Centres of Excellence funding is available to all, but those who propose to conduct research likely to create intellectual property have a decided edge.

Universities, Applied Biotechnology, Intellectual Property, and the Power to Profit and Survive

Universities, like government, have mixed views regarding intellectual property and applied biotechnology. Universities are under increasing pressure to satisfy the sort of accountability demands pressed on governments, and intellectual property plays an important role in demonstrating that investment in universities returns social and economic benefits. Intellectual property is sometimes thought to provide a measure of the broader social relevance of university research: we know that universities conduct **socially relevant research** when the conclusions of that research are eligible for legal protection and sale in ways that can generate **royalty revenue** for the university. In some views, this represents a desirable shift in university's attitudes, as patenting of research results formerly put into the public domain via publication may amount to little more than finally profiting fairly from the results of researchers' labours.

Not all is rosy within the university, however. The expansion of university technology transfer offices is frequently met with the criticism that universities are **pandering to industry** and, in doing so, are **fracturing the mission** of the university as a research and teaching organisation dedicated to the public good. Some researchers are concerned that university-industry relations amount to little more than poorly targetted handouts to industry, as funding bodies promoting these relations are seen to focus on numbers of patents and licensees rather than the net economic and social impact of particular inventions. Still other researchers may have no particular attitude at all toward intellectual property. While many university-faculty agreements include intellectual property production as 'research' to be counted toward promotion, this formal recognition remains relatively unimportant within the culture of university research. The old slogan 'publish or perish' remains true today. Teaching and service must exceed a minimum performance threshold, yet the key to prestige and **promotion lies in publication**, and publication is rarely possible without funding to support research leading to publication. In this context, intellectual property considerations are often viewed as a hindrance: yet one more administrative demand pulling researchers away from the lab bench or out of the field to fill out paperwork, and a potential **barrier to timely publication**, the 'real' and important output of scientific research leading to promotion, security, and prestige.

"In fact, there may be organisations whose stock of intellectual capital makes them so competitive that there may be little point to their patenting a product or process which can easily be withheld from competitors as a trade secret."

Private Sector, Applied Biotechnology, Intellectual Property, and the Power to Compete

Receptors, end-users, entrepreneurs, or simply business operators all want research results for one reason: to **improve business competitiveness.** Research results are only of interest to the extent that they solve a problem that consumers will pay to have solved, and intellectual property rights are important only to the

extent that they limit competitors' free access to that solution. The cost of acquiring and maintaining intellectual property rights is simply another cost to be considered: possession or lack of possession of intellectual property is not a sign or cause of success or failure as a biotechnology business. The end user is not concerned with economic development of a sector, public accountability, or publication or even prestige that is not directly connected to securing a competitive advantage in the marketplace.

Does any one of government, universities, researchers, or business operators have the right view of the importance of intellectual property rights in applied biotechnology R&D? Closer attention to intellectual property rights reveals that each group has only a partial picture, and each group will benefit from taking a wider view of the place of intellectual property in applied biotechnology research, development, and commercialisation.

What Intellectual Property Is and Is Not

'Intellectual property' is often used informally to describe a wide range of ideas, practices, and technology. While this broad use helpfully identifies intangible ideas as the sort of things that can be property, informal usage falls short of identifying specific categories of legal property giving legally enforceable rights to owners. University intellectual property or technology transfer offices are most concerned with **patents** and **licensing** use of patented products or processes. Patents are certainly important to applied biotechnology, yet they are not the only relevant form of intellectual property. **Copyright** is also important to researchers, and not just in control of reproduction of published research results. Bioinformatics research often produces software eligible for copyright protection. **Plant Breeders' Rights** may be increasingly important, as, for example, plant-derived lipids may gain use in fish feeds. Some aquaculture know-how transferred from university researchers to aquaculturists may be usefully protected as **trade secrets** and the brand or tag phrase by which a product or process is known may be usefully protected by **trademarks.** There are other, less relevant kinds of intellectual property, yet even this quick sketch shows that there is more to intellectual property and aquaculture biotechnology than counting patents. We will continue to focus on patents here since they dominate discussion of intellectual property in aquaculture biotechnology.

A patent is a legally enforceable property right in a product or process and not just an abstract idea. That product or process must be accepted by the Patent Office of the Canadian Intellectual Property Office as new, useful in the sense that it is actually functional, and involving an inobvious or inventive step forward. Perhaps the most important fact aquaculturists should know about patents is that the patent-granting process contains no test for and no guarantee of the **scientific merit** of the patented product or process. The utility, or usefulness test for patentability inquires into the in principle feasibility of the product or process, yet this process almost never requires the building of a **prototype**, and never requires testing for **safety,** evaluation of the **legality** of using the product or process in a particular place, or testing for **marketability** of the patented product or process. Note also that **'patent pending'** means simply that a patent has been applied for and is no guarantee of patentability or commercial viability. The Canadian Patents Database contains various applications for patents on perpetual motion machines. One application, for example, proposes to patent a 'cosmic cube' computer purportedly capable of producing "cubical wavefields of ultrapowerful energy by subtracting time and space from the human equation."

Fortunately, there are plenty of patents that exceed the minimum set by the utility requirement, and many patents are at the root of successful commercial products and processes. Yet even good patented ideas are **not instant cash**. Stanley Cohen and Herbert Boyer of Stanford University patented recombinant DNA technology in 1974, generating in the region of $100 million in royalties, the basis of Genentech Inc., and 200 licenses sold to other firms. One hundred million is a lot of money, but it is not an unimaginable sum, and that was a very special patent opportunity. Even if you are fortunate enough to find a good idea worth stealing, and for that reason worth protecting, there is the question of whether that idea is worth the **cost of filing** and securing a patent (up $50,000 US dollars to file in the US) and further costs as patents are sought in other jurisdictions. It is worth noting in this context that there is **no 'global patent',** not even a pan-European patent. Smart strategy suggests patenting in the largest markets for your product or process, and that step requires someone to pay for it. Once a patent is issued—and there is no guarantee of that—there is still further work to be done. There are no 'patent police' in Canada or the US: the patent holder must take action to prevent unauthorised use of the patented product or process. **Defence of patent rights** can be expensive; the University of Colorado Health Sciences Center recently won a $58.3 million patent infringement suit against pharmaceutical firm Wyeth, but that victory cost $28.3 million and took 11 years to resolve.[7]

". . . effective knowledge transfer, commercialisation and competitive advantage can often be secured without the effort and expense associated with patents."

It is worth adding a few practical observations about what is omitted when IP is taken to be the measure of successful innovation in aquaculture biotechnology. Many funders appear to suppose that intellectual property (particularly patents) can be generated by university researchers who transfer that intellectual property to the private sector, then return to the lab bench to create still more intellectual property. While this may sometimes occur, the process is often more complicated. Patents are **not self-managing**: someone must handle licensing agreements, and someone must manage license revenue. Development of products or processes may also require negotiation of agreements for transfer of **advice regarding implementation of the patent.** Patents are not always easily transferred bright ideas boosting the productivity of any who take advantage of them. Successful commercialisation may require extensive and continued involvement of researchers in long and difficult periods of testing and adjustment. Patents are not themselves a measure of this **intellectual capital** or problem-solving capacity possessed by the researchers or organisation. In fact, there may be organisations whose stock of intellectual capital makes them so competitive that there may be little point to their patenting a product or process that can easily be withheld from competitors as a **trade secret**. Some ideas may be so complex in application that the organisation has a **lead time advantage** in getting to market ahead of competitors who lack necessary experience and intangible know- how. Unregistered, free trade secrets protecting vital know-how may contribute to innovation and competitiveness without ever being counted. Similarly, there may be little point to patenting very specific **local solutions** to local problems not encountered by competitors, or small, **incremental advances** vital to competitiveness yet not financially worth protecting via the patent process. Finally, there may be no point to patenting a product or process whose **free widespread public availability is desirable** in order to sustain an entire industry. The discoverer of a PCB-free source of feed, for example, might wish for it to be freely available to help preserve an otherwise unsustainable part of the aquaculture industry in which the researcher has a particular interest.

Cultural Change and Knowledge Management Programs

If there is any single 'take home message' from the preceding discussion it is that intellectual property is of variable relevance and importance to participants in the research-to-innovation cycle. It is worth taking care to ensure that what appear to be disagreements over intellectual property are not quite different disagreements—often about the kind of cultural change needed on all sides to enable meaningful collaboration. Intellectual property is most likely to serve a useful role when partners agree that innovation is not reducible to production of intellectual property, and intellectual property relevant to innovation is not reducible to patents. Organisations collaborating to use biotechnology to solve problems in aquaculture may be more successful in achieving their goals if they recognise instead that innovation is driven by intellectual wealth of the kind discussed in preceding sections, of which intellectual property is just a part.

AquaNet, the Network of Centres of Excellence in Aquaculture, has begun to develop a **knowledge management program** aimed at capturing the knowledge flows which make up intellectual wealth. The program will set a common process for internal reporting of research results, and for demonstrating to funders and stakeholders that AquaNet creates and transfers various forms of knowledge to the aquaculture sector. Success indicators include measurement of intellectual property production, and such easily measured activities as troubleshooting, training of end-users, and adaptation of products and processes to local needs. Most importantly, a knowledge management program allows all partners in an aquaculture biotechnology R&D cycle to use intellectual property rights to best advantage. Patents are undeniably an important tool in the research-to-innovation cycle, yet demonstrably effective knowledge transfer, commercialisation and competitive advantage can often be secured without the effort and expense associated with patents.

Resources

- Research, Technology and Innovation section of Industry Canada's business and consumer website: http://www.strategis.gc.ca/sc_innov/engdoc/homepage.html?categories=e_res
- Canada's Biotechnology Strategy: http://biotech.gc.ca
- Canadian Intellectual Property Office: http://cipo.gc.ca/

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3. http://biotech.ic.gc.ca/epic/internet/incbs-scb.ns/en/by00217e.html (accessed June2, 2004)
4. http://biotech.ic.gc.ca/epic/internet/incbs-scb.ns/en/by00213e.html (accessed June2, 2004)
5. http://www.nserc.ca/fact_e_htm ((accessed June2, 2004)
6. Consider, for example, patent applications 227901, 2213599,2200361, 200302. The Canadian Patents Database is viewable at http://patents1.ic.gc.ca/intro-e.html (accessed June 2, 2004)
7. http://chronicle.com/prm/weekly/v50/i34/34a02801.htm (accessed June 2, 2004)

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The Role of Molecular Biology in International Regulation: Shellfish Health

Kimberly Reece

Kimberly S. Reece and Eugene M. Burreson

Molecular diagnostics are now commonly accepted and employed in standard assays for aquatic animal disease surveys and certifications for trade. The Office International des Épizooties (OIE) develops internationally recognized standards for animal pathogen diagnostic protocols. OIE objectives include collecting and disseminating information on animal diseases around the world, and ensuring the sanitary safety of international trade in animals and animal products. OIE identifies diseases of concern, called "reportable diseases," and develops measures to prevent disease transfer while trying to avoid unjustified barriers to trade. OIE approves a Reference Laboratory for each reportable disease, which is responsible for developing and standardizing diagnostic tests and providing diagnostic support for international colleagues. Diagnostic methods developed by reference laboratories are published in the *Manual of Diagnostic Tests for Aquatic Animals.* The manual now includes molecular protocols for presumptive and confirmatory diagnosis of several aquatic pathogens. Molecular assays can facilitate rapid diagnosis, which is critical to prevent spread of pathogens. However, it is essential that these protocols are reliable and accurate. Interpretation and confirmation of results from molecular assays can be complex. Adequate molecular information is needed for development of rigorous assays and the protocols should be validated against established diagnostic procedures.

Office International des Épizooties

The OIE is a Paris-based organization whose aim is to assure the sanitary safety of international trade in animals and animal products. The OIE publishes three documents specifically dealing with aquatic animals, which include information on fish, molluscs and crustaceans —1) the *Aquatic Animal Health Code* that lists all reportable (notifiable) diseases and explains the steps necessary to be designated a disease-free country, and the reporting requirements should a disease outbreak occur; 2) the *Manual of Diagnostic Tests for Aquatic Animals*, which includes OIE-approved diagnostic methods and standards developed by the reference laboratories; and 3) *Annual Reports of OIE Reference Laboratories,* which document all research and diagnostic support provided by the reference laboratories. In addition to a Reference Laboratory, a Reference Expert, who is responsible for providing advice to the OIE, is appointed for each listed disease. Reference Laboratories serve as an identification resource for researchers and government agencies around the world and are also obligated to develop and standardize diagnostic protocols for relevant disease agents. Most countries require confirmation

of identification of reportable disease outbreaks by the OIE Reference Laboratory for that disease agent. For identification of aquatic pathogens and diagnoses, OIE laboratories are increasingly using molecular detection protocols, as they can be very rapid and sensitive. However, as discussed below, limitations of molecular techniques need to be understood and assays should undergo thorough testing.

Advantages and Limitations of Molecular Diagnostics

Developments in molecular genetic technology and the proliferation of DNA sequence information during the past twenty years have facilitated progress in aquatic pathogen detection and identification. There is an ever-expanding database of molecular information available, which can be utilized for development of sensitive and specific detection assays. Molecular diagnostics, including DNA probes for in situ hybridizations and primers for use in the polymerase chain reaction (PCR) are now available for a wide a variety of pathogens found in the aquatic environment (for review see Cunningham[1]). These detection methods can greatly facilitate disease diagnoses, especially where it is difficult and/or time consuming to isolate and identify pathogens, and in situations where pathogens cannot be easily distinguished based on morphological characters. In addition, molecular diagnostics are invaluable when a particular species within a genus, or even certain strains of the same species, may be pathogenic while closely related species or strains are harmless.

". . . non-specific amplification can occur in PCR assays. . . . This can be especially problematic when the targeted species or strain is pathogenic, while close relatives are harmless. Such false positives can lead to unnecessary trade restrictions."

Although molecular techniques can be very powerful, they must be designed and used appropriately. The intended application should be considered before assay development. It is important to realize, for example, that PCR assays detect the presence of an organism's DNA and do not necessarily indicate that a viable pathogen, capable of causing disease, is present. This fact can create difficulties when trying to assess the risk indicated by positive assay results.[2] PCR assays, however, can be very helpful when large numbers of animals need to be screened in a short period of time and for environmental monitoring of a pathogen. More confidence in the relevance of PCR results is possible in situations where positive assays occur in conjunction with observation of a pathogen and host tissue response in histopathological sections, or with positive in situ hybridization or immuno-assay results.

In situ hybridization DNA probes or antibodies may be more useful than PCR primers for indicating the viability of pathogens. Strong positive signals are obtained when labeled in situ DNA probes bind to an RNA target, as well as DNA, of the pathogen in histological sections. RNA is only produced in actively metabolizing cells and is relatively unstable compared to DNA, turning over frequently in the cell and quickly degrading after cell death. Antibodies for immunoassays often target antigens on the surface of a pathogen cell and may be degraded, or undergo conformational changes that preclude antibody binding when the cell dies.

In addition, non-specific amplification can occur in PCR assays, even after extensive specificity testing has been done in the laboratory. Amplification of DNA from an organism closely related to the target species or strain can occur, particularly from environmental samples, if close relatives have not been recognized and characterized. This can be especially problematic when the targeted species or strain is pathogenic, while close relatives are harmless. Such false positives can lead to unnecessary trade restrictions. Increased confidence in the identity of an amplification product can be obtained by doing restriction fragment length polymorphism analysis (RFLP) of the product, and ultimately DNA sequencing of the amplicon can be done to verify that the target was amplified.

Molecular assays are likely to be more useful and specific when adequate molecular information is available. This information is DNA sequence data for designing PCR primers and DNA probes. Development of genus-specific, species-specific and/or strain-specific DNA probes and PCR primers, for example, entails obtaining sequence data for an intended target locus from as many different strains and species within a genus as possible. In addition, DNA sequences from targeted loci are needed from closely related taxa. Intra- as well as inter-specific sequence variation needs to be examined and adequately characterized. This approach optimizes the chance of developing a specific probe, for example, that works for all members of the genus, species or strain (inclusivity), and that does not cross-react with members of other genera, species or strains (specificity). To minimize the chances of a species-specific probe failing to detect a particular strain of a species, as many strains as possible from a wide geographic range, and in some cases the host range, should be examined.

Shellfish Pathogen Molecular Diagnostics

PCR assays for various *Perkinsus* species—protozoan parasites of marine mollusks—illustrate the need for obtaining adequate data from multiple strains and species. The small subunit (SSU) ribosomal RNA gene is often the target for shellfish pathogen diagnostic PCR assays. Among *Perkinsus* species, however, the sequences of SSU genes are highly conserved, precluding it as a target for species-specific assays. The internal transcribed spacer (ITS) region, on the other hand, is variable and the individual species can often be identified by comparison to GenBank deposited ITS sequences for *Perkinsus* species.[3] Several species-specific PCR assays have been developed based on sequences unique to each *Perkinsus* species, and most of the species-specific primers target the ITS or non-transcribed spacer (NTS) regions of the ribosomal RNA gene complex.[4-11] Within the ITS region intra-specific variation has been observed for several *Perkinsus* species. Fourteen polymorphic nucleotide sites were detected following sequence analysis of 84 ITS region DNA clones from a total of 12 *P. marinus* isolates[12] resulting in sequence similarity ranging from 98.49% to 100%. These polymorphisms illustrate the need for caution when designing species-specific probes and PCR primers. Primers that target regions with polymorphic sites may fail to amplify all of the genetic strains within a species. Although the NTS region is also targeted by many *Perkinsus* species' assays,[6,7, 9,10] the intra-specific sequence variation within the NTS region, unlike the ITS region, has not been broadly assessed so that there may be a risk of false-negatives due to polymorphisms among the NTS sequences of genetic strains within each species. In order to develop inclusive, yet specific, molecular diagnostics for *Perkinsus* species, large DNA sequence sample sizes, including sequences from multiple clones from multiple isolates across a wide geographic and/or host range, if applicable, are necessary. These data will allow detection of nucleotide variations present within the target locus and design of probes and primers that target sequences that are conserved within a species, but vary among species, so that the chance of obtaining false negatives or false positives is minimized.

Appropriate identification and discrimination of *Perkinsus* species has been problematic. Molecular information is being used to facilitate recognition of described and new species. Identification and designation of the various species is needed for molecular diagnostics to be developed and applied appropriately. Historically, *Perkinsus* species descriptions have relied largely on differences in geographic and host range. Although slight variations in morphology have been ob-

"To minimize the chances of a species-specific probe failing to detect a particular strain of a species, as many strains as possible from a wide geographic range, and in some cases the host range, should be examined."

served, it is difficult to discriminate among species based on morphology alone.[13-18] In addition, individual *Perkinsus* species cannot be differentiated using conventional diagnostic techniques such as histological examination and the Ray's fluid thioglycollate (RFTM) assay.[19] With the relatively recent availability of DNA sequence data for these organisms, some previous species designations have been questioned. For example, the percent sequence similarity found between ITS sequences deposited in GenBank for *P. olseni* and *P. atlanticus* is very high (98.8% to 100%) and greater than that observed among some *P. marinus* ITS sequences. Based largely on DNA sequence data, these two species have recently been synonymized by Murell et al.[10] There is also evidence from DNA sequence data that *P. chesapeaki* and *P. andrewsi* are the same species. Distinct internal transcribed spacer (ITS) sequences that were previously attributed to these two different species have been found together in several clonal cultures isolated from multiple hosts in the Chesapeake Bay region of the USA. This suggests that the observed polymorphisms demonstrate intra-specific, rather than inter-specific, variation[20] and that *P. chesapeaki* and *P. andrewsi* should be synonymized. On the other hand, the *P. qugwadi* ITS sequence is quite divergent from that of the other *Perkinsus* species. Based on the DNA data, and the fact that *P. qugwadi* cells do not enlarge in RFTM, some have suggested that it may not be a valid *Perkinsus* species.[21] The intended target genus, species or strain must be known and discriminated so that the necessary molecular data can be obtained. In some cases, however, the full geographic or host range of the target organism may not be known and as more information and molecular data become available it may be necessary to modify molecular diagnostics so that they demonstrate the intended inclusivity and specificity.

". . . to confidently employ molecular tools in disease diagnoses, the assays should undergo rigorous validation against established diagnostic protocols."

Finally, in order to confidently employ molecular tools in disease diagnoses, the assays should undergo rigorous validation against established diagnostic protocols. Yarnall et al.[8] developed a quantitative PCR assay for *P. marinus* that proved to be more sensitive than the conventional RFTM assays of tissues and hemolymph, and comparable in sensitivity to the body burden RFTM assay described by Fisher and Oliver.[22] Both the molecular and body burden assays detected the presence of *P. marinus* in the same number of individuals,[8] with the PCR allowing the quantification of the parasite DNA present in the host.

PCR primers[23] and a DNA probe[24] for another protozoan oyster parasite *Haplosporidium nelsoni* have been extensively tested for sensitivity and specificity. These diagnostics have been used to identify *H. nelsoni* in oysters around the world[25-27] and recently confirmed that *H. nelsoni* was the cause of epizootic oyster mortality in Nova Scotia, Canada.[28] Another closely related, yet less pathogenic haplosporidian, *H. costale,* is also found in oysters along the US east coast. *Haplosporidium nelsoni* and *H. costale* are extremely difficult to differentiate based on morphology of plasmodia. However, DNA probes and PCR primers specific to each parasite species can be used for differential diagnosis and even have demonstrated mixed infections in oysters.[29]

Cochennec et al.[30] and Carnegie et al.[31] both developed PCR assays for a haplosporidian microcell parasite of flat oysters, *Bonamia ostreae.* At the time, the assays were thought to be *B. ostreae*-specific. However, more recent sequence data from newly identified species in the genus *Bonamia* suggest that the assays are actually broader in specificity. Based on target DNA sequence similarity, each assay should detect two or more species in the genus.[32] Both assays have undergone some validation against histocytological diagnosis with both detecting almost four times more *B. ostreae* infections than histocytology.[31,33]

Molecular diagnostics for the hard clam *(Mercenaria mercenaria)* parasite QPX were validated by comparison with histological diagnosis of over 200 field samples.[34] It was found that PCR assay sensitivity was improved by subjecting any initially negative PCR products to a second round of amplification. In addition, the sensitivity of oligonucleotide DNA probes was greatly improved by using a cocktail of two different probes that were specifically designed to target the SSU gene of QPX. Overall we have found that nested primers, reamplification, and/or touchdown PCR and oligonucleotide probe cocktails can in many cases improve sensitivity and specificity of molecular assays.

General Recommendations

In conclusion, in developing and implementing molecular diagnostics it is important to obtain as much sequence data and information as possible from the targeted pathogen and any known related organisms. We must recognize that assays may need to be modified over time as new species are identified and more molecular data become available. It can be helpful to develop multiple assays for a particular pathogen so that cross-confirmation of results is possible. Even more confidence in results can be obtained if probes and primers for these assays are designed to target different regions of the organism's genome and are used for cross-validation of results.

"It can be helpful to develop multiple assays for a particular pathogen so that cross-confirmation of results is possible. Even more confidence in results can be obtained if probes and primers for these assays are designed to target different regions of the organism's genome."

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Detection and Characterization of Nodavirus in Several Marine Fish Species from the Northeastern Atlantic

Nellie Gagné

N. Gagné, S. C. Johnson, M. Cook-Versloot, A.-M. MacKinnon, and G. Olivier

Nodaviruses are responsible for causing disease outbreaks, mainly in hatchery-reared larvae and juveniles, of a wide variety of fishes throughout the world. In the Atlantic Provinces of Canada, the first case of disease caused by nodavirus was reported in 1999 in juvenile Atlantic cod being reared in Nova Scotia. More recently, disease outbreaks have been identified in hatchery-reared Atlantic cod and haddock in Newfoundland and New Brunswick, respectively, and along the east coast of the United States. Nodavirus has also been recovered from wild Atlantic cod and winter flounder. The virus coat proteins (capsid) of nodavirus isolates obtained from various fish host species were partially sequenced. Data indicate that all the nodaviruses isolated from eastern North America were closely related to one another but distinct from the European isolates already sequenced. At the protein level, differences in coat protein sequences were seen only for strains isolated from Atlantic cod originating from Newfoundland. Our results suggest that fish nodaviruses (NNV) in Atlantic Canada have evolved for some time to form a monophyletic group, distinct from other isolates found in coldwater species.

Nonlethal methods for detection of nodaviruses are necessary to develop management strategies for this disease. Based on the results of this study, new primers were designed and developed for an improved RT-PCR assay able to detect North Atlantic nodaviruses in ovarian fluid, eggs, blood and other tissues.

Introduction

Fish nodaviruses (NNV) belonging to the genus *Betanodaviridae* have been reported to cause disease in over 30 species of marine finfish used in aquaculture worldwide. The disease is known either as viral nervous necrosis (VNN), viral encephalopathy and retinopathy (VER), or fish encephalitis. NNV outbreaks result in high mortalities in hatchery-reared larvae and juveniles, but adults can also be affected.[8] In North America, outbreaks of disease caused by nodavirus have been reported in hatchery-reared Atlantic cod *(Gadus morhua)* and/or haddock *(Melanogrammus aeglefinus)* in Newfoundland, Nova Scotia, New Brunswick and along the east coast of the United States. These disease outbreaks have resulted in high levels of morbidity and mortality.[6] The presence of nodavirus in

wild adult Atlantic cod and wild adult winter flounder *(Pleuronectes americanus)* has been reported from Atlantic Canada.[2,3] In Europe, nodavirus infection has been reported in cultured juvenile Atlantic cod from the UK with low mortality rates (approximately 2%) observed over a 3-month period.[12]

Nodaviruses are small non-enveloped viruses, and their genome consists of 2 sense RNA strands. RNA1 (3.1 kb) encodes a putative RNA-dependent RNA polymerase (RdRp) and the RNA2 (1.4 kb) encodes the capsid protein precursor .[7] To date, comparisons between the nucleotide sequences of a variable region of the capsid protein referred as T4[9] has led to the division of the genus *Betanodavirus* into 7 type species: barfin flounder nervous necrosis virus (BFNNV), *Dicentrarchus labrax* encephalitis virus (DIEV), Japanese flounder nervous necrosis virus (JFNNV), *Lates calcarifer* encephalitis virus (LCEV), redspotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), and tiger puffer nervous necrosis virus (TPNNV).[15]

To develop appropriate disease management strategies for this disease there is a need to: 1) determine whether the NNV isolates obtained from different hosts species are the same, 2) develop improved and validated methods for NNV screening and disease diagnosis, and 3) develop non-lethal methods for screening broodstock. With respect to screening and disease diagnosis, reverse-transcriptase polymerase chain reaction (RT-PCR) assays for NNV can be conveniently used to check the health status of animals by testing reproductive material, eggs, larvae and juveniles at all stages of production.[14] Although RT-PCR methods for the detection of nodaviruses are available,[5,9] their sensitivity depends greatly on having primers that are designed to complement the NNV isolates of interest.

In this study, we partially sequenced the coat proteins of NNV isolates from eastern Canada and northeastern USA to determine the phylogenetic relationship between these isolates and isolates from other host species and geographical regions. Based on the results, RT-PCR primers were designed and used to optimize an RT-PCR assay for these isolates.

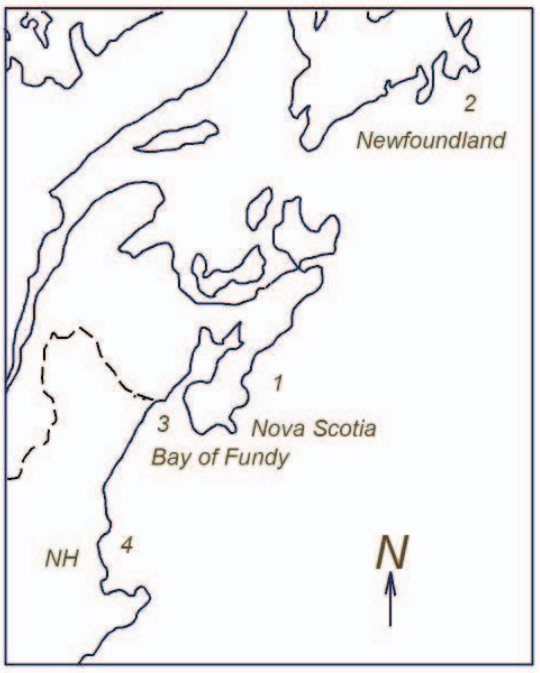


Figure 1
Approximate location of fish aquaculture facilities or fish capture sites where nodaviruses were isolated: 1) Atlantic cod isolates from Nova Scotia; 2) Atlantic cod isolates from Newfoundland; 3) wild winter flounder isolates from New Brunswick; and 4) Atlantic cod isolates from New Hampshire.

Methods

NNV isolates

Isolates of NNV were obtained from disease outbreaks and surveys of wild fish from a variety of locations in eastern Canada and northeastern USA (Fig. 1). All isolates were obtained using the methods outlined below for haddock tissues and were kept at -70°C for long-term storage.

Cell culture of nodavirus

Immediately after necropsy, haddock brain and eye tissue were processed for viral culture either individually or as pools of tissues from up to 5 individuals. Briefly, tissues were homogenized in a dilution of 1:50 w/v in Hanks' balanced salt solution (HBSS) pH 7.6 using a stomacher (Seward Laboratory). Homogenates were centrifuged at 2500 x g for 15 min at 4°C and resulting supernatants were aseptically filtered through 0.45 m pore diameter membranes. Filtrates (0.1mL) were applied in duplicate to 24-well plates containing striped snakehead-1 (SSN-1) cells (ECACC#96082808) and cultured at 25°C for a minimum of

28 days. Cell cultures showing CPE were sub-cultured and the presence of NNV was confirmed by RT-PCR following the method described below. Isolates obtained from this work were named Had02ac1, Had02ac2, Had02ac3 (haddock, Bay of Fundy), Wf00ac (winter flounder, Bay of Fundy), Cod02ac (cod, Nova Scotia), Cod02ac2 (cod, Newfoundland) and Cod02ac3 (cod, New Hampshire).

Isolates of NNV obtained from other laboratories were re-inoculated onto SSN-1 cells and cultured at 25°C for a minimum of 7 days prior to RNA isolation.

RNA extraction and RT-PCR

RT-PCR was used to amplify coat protein sequences of NNV from haddock tissues and from cell culture lysates. Total RNA was isolated from cell culture lysates and RNALater® preserved eye and brain tissues using TRI Reagent™ (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. Resulting total RNA samples were dissolved in 20 L each of sterile H_2O containing RNase inhibitor. Reverse transcription primed with random hexamers was done with a RevertAid™ First strand cDNA synthesis kit (MBI Fermentas Inc., Burlington, ON) using up to 5 g of RNA and following manufacturer's instructions. PCR amplifications were done with AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Foster City, CA) in 25 L volumes containing 2 L of cDNA, 0.4 M of each primer, 1 L of BSA 1% and 2.5 mM $MgCl_2$. PCR conditions were 94°C for 4 min (initial denaturation), followed by 10 cycles of [94°C for 30 s, 60°C (-1ºC per cycle) for 30 s, 72°C for 90 s] and then 35 cycles of [94°C for 30 s, 50°C for 30 s, 72°C for 90 s] and a final hold at 72°C for 5 min. Amplification products were resolved on 1.5% agarose gels.

Several primers designed to amplify NNV coat protein sequences were used in this study (Table 1). Primers 313F and 591R were used to identify NNV in haddock tissues and cell cultures, as well as in RT-PCR optimization studies. Primer 495R was designed for use in RT-PCR optimization studies. Primers 1322R, NVHF and NVH-R2 were designed to amplify larger regions of the coat protein sequence for use in phylogenetic studies.

RT-PCR optimization

The sensitivity of RT-PCR using either primers 313F/591R or 313F/495R was compared using the PCR conditions outlined above. The effect of RNA denaturation at 95°C for 5 min and at 70°C for 10 min was also compared to evaluate the effect of denaturation temperature on the sensitivity of RT-PCR detection. Sensitivity was evaluated on serial dilutions in PBS of NNV cultures. Briefly, a NNV culture was diluted 1/10 in PBS before extracting RNA as outlined above. RNA denaturation was done either at 70°C or 95°C before reverse transcription. PCR amplifications of the NNV serial dilutions were done using either primers 313F/591R or 313F/495R and the method described above.

As the tissue type may be a limiting factor in RT-PCR, we tested our optimized RT-PCR procedure on samples of haddock eggs that were spiked with virus. Haddock eggs (1 mL vol-

Table 1. Summary of primers used to amplify NNV. Numbering refers to the primer position on NNV sequences from Atlantic halibut (accession number AJ245641).

Primer Name	Direction	Primer Sequence
313F	Forward	5 aat tca gcc aat gtg ccc cgc aaa 3
591R	Reverse	5 cag cga cac agc acc gac acg 3
495R	Reverse	5 acc gag gtc cag agg agt gcg 3
1322R	Reverse	5 cgg taa ccc aac aag ccc aaa ga 3
NVHF	Forward	5 aat ggt acg caa agg tga 3
NVG-R2 (1349R)	Reverse	5 gcg gtg gtg ctc wct rcg cgg 3

ume) were mixed with 1mL of a 1:1000 dilution of NNV positive cell lysates and left at room temperature for 30 min. The eggs were then washed twice with 8 volumes of PBS and RNA was extracted from the eggs as described previously, followed by RT-PCR analysis using primers 313F and 591R.

Partial sequencing of the coat protein

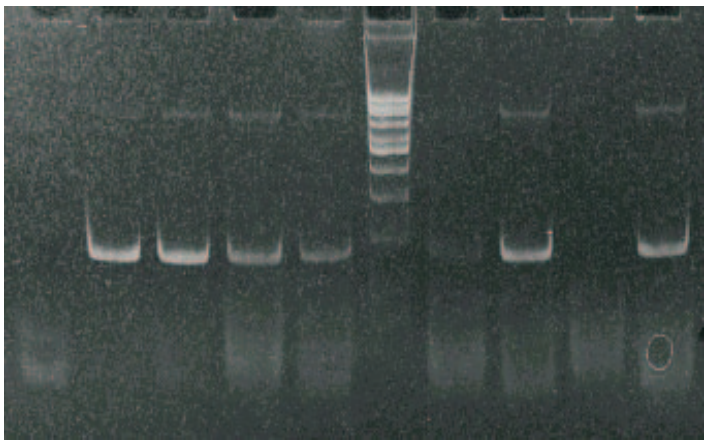
Partial coat protein sequences were obtained from RT-PCR products using primer sets 313F/1322R or NVHF/NVH-R2 and conditions as described above. Internal primers were also used for sequencing these fragments. RT-PCR products were either sequenced directly or after cloning with TOPO TA cloning® kit (Invitrogen, Burlington, ON) following manufacturer's instructions.

Each isolate was sequenced at least twice, and on both strands. Sequencing was done either on an ABI 373 or an ABI 3100 Automated Sequencer using PRISM Big Dye Terminator (PE Applied Biosystems, Foster City, CA) or on an ALFexpress II using Thermo Sequenase Cy5 dye terminators (Amersham Biosciences, Baie d'Urfé, QC).

Phylogenetic analysis

Sequences were assembled using GeneDoc (http://www.psc.edu/biomed/genedoc). A BLAST similarity search[1] for significant homologous sequences was done against GenBank® (National Center for Biotechnology Information) using the NNV sequence of the Newfoundland Atlantic cod isolate. Other NNV coat protein sequences representing all possible type species were selected for phylogenetic analysis and sequences were retrieved from Genbank (AF445800 (Cod99AC), AJ245641 (HhNNV1), AF160473 (HhNNV2), D38635 (BF93Hok), D30814 (SJOri), U39876 (Diev-F), D38637 (TP93kag), D38636 (RG91tok), D38527 (JF93Hir), AF499774, AF245004, AF245003, AJ277811, AF175518, AJ277810, AF283554, AB045980, AF534998, Y08700, AF318942, NC02037). Multiple alignments of nucleotide and deduced amino acid sequences were performed using ClustalX (1.81).[13] Phylogenetic analysis was conducted with DNAML from the PHYLIP package (3.6a3)[4] and with TREE-Puzzle (5.0).[11] TreeView version 1.6.6[10] was used to view the trees. Isolates Ba94aus sequence was obtained from Nishizawa et al.[16]

Figure 2
Electrophoresis on acrylamide (10%) of RT-PCR products from diluted NNV cell lysates. Lane 1, negative control; lane 6, DNA ladder. Primers are 313F and 495R. Lane 2 to 5, 7 and 9, dilutions 10^0 to 10^{-5} with RNA denaturation at 70°C; lanes 8 and 10, dilution 10^{-4} and 10^{-5}, RNA denaturation at 95°C. No PCR product was observed beyond a dilution of 10^{-4} at 70°C RNA denaturation, but would still be visible at 10^{-6} on average at 95ºC RNA denaturation.



Results and Discussion

Isolation and confirmation of NNV in haddock

In 2002, juvenile haddock (3-6 months old) that were being held at the Biological Station in St. Andrews began showing signs of disease similar to that reported for other species affected by VNN. A total of 164 juvenile haddock were submitted for disease diagnosis. Samples were examined by virus culture using both individual and pooled samples of brain and eye tissue homogenates and by RT-PCR on eye and brain tissue. RT-PCR was conducted using primers 313F and 591R to confirm the presence of NNV in CPE positive cell cultures. In total, 74 (82%) out of the 90 samples were positive for NNV by culture and all were confirmed as NNV positive

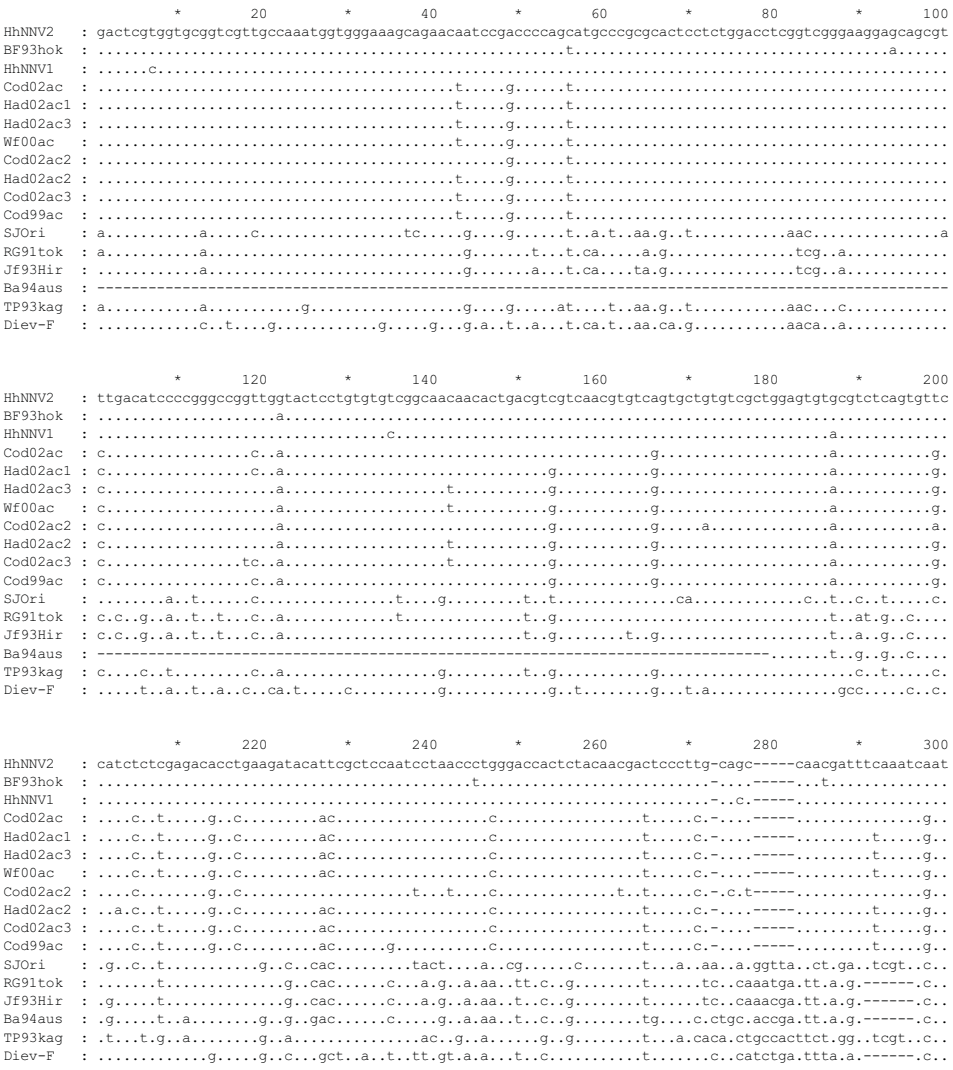
by RT-PCR. Remaining samples were tested by RT-PCR only using the primers 313F and 591R. Of the 146 samples of brain and eye tissue examined, 104 (71.5 %) were positive for NNV. For 61 individuals for which we had both cell-culture and RT-PCR data, we compared the results obtained by these two methods. Only 1 sample that was positive by cell culture was not detected by RT-PCR and 7 samples found to be positive by RT-PCR were negative by cell culture.

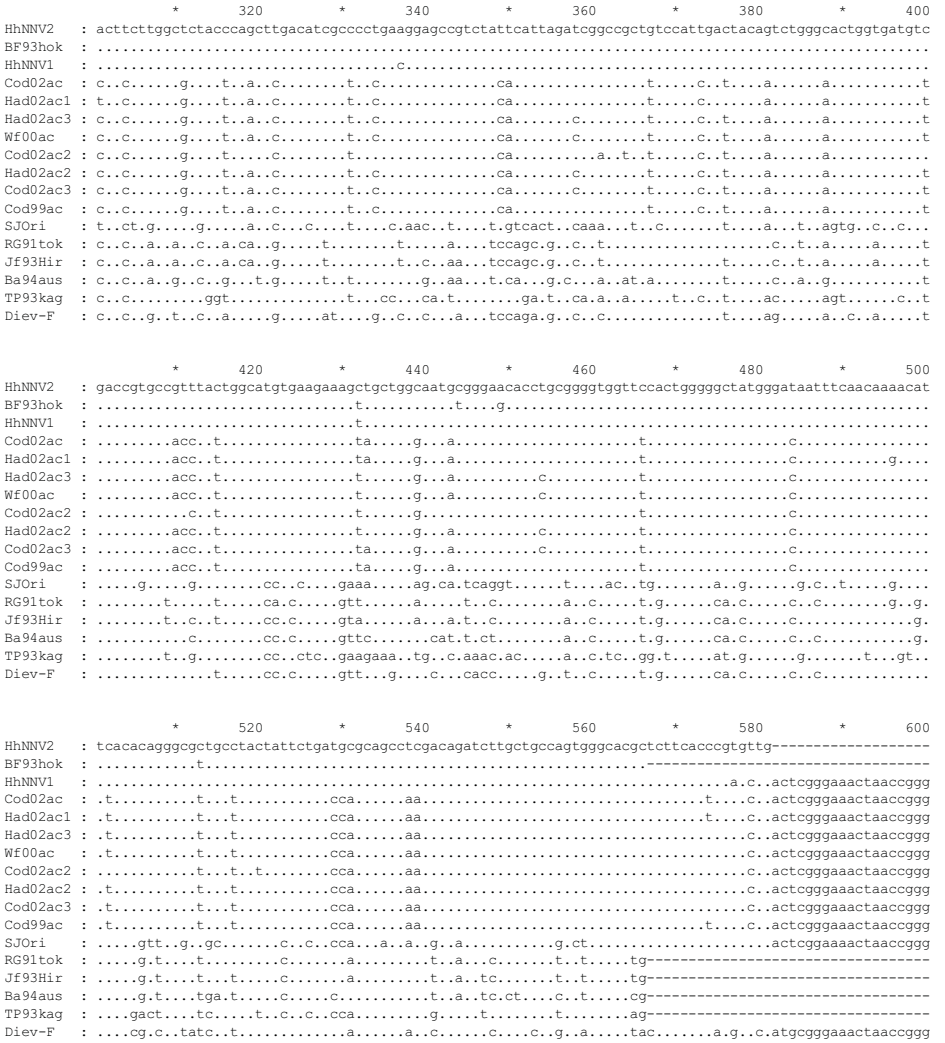
RT-PCR optimization

Denaturation of RNA prior to reverse transcription at 95°C improved the sensitivity of the RT-PCR assay by 2-3 log when compared to the temperature of 70°C (Fig. 2) suggested by the manufacturer. The primer sensitivity and specificity was

Figure 3

Multiple alignment of the partial coat protein nucleotide sequence of the North Atlantic NNV isolates, type species, and barfin flounder clade members. The alignment corresponds to position 446 to 1047 of SJOri (GenBank accession no. D30814), and covers the variable region T4 (position 160 to 586 of this alignment). Sequences were aligned using Clustal X. Gaps are represented by dashed lines.





tested using serial dilutions of cell lysates. We could detect, on average, the equivalent of 10 to 100 TCID50 using primers 313F/495R and a temperature of 95°C for RNA denaturation. Haddock eggs exposed to NNV were also consistently found positive with the optimized RT-PCR assay or with primers 313F /591R (results not shown). Our primer 313F perfectly matches the sequences of all the NNV isolates used in the phylogenetic analysis and represents a good "universal NNV primer". The sequence of primer 591R matches most of them, with at most 3 nucleotide differences with SJOri (accession number D30814). Primer 495R matches the sequences of all isolates within the barfin flounder clade, as well as the sequences of isolates described in this study.

Partial coat protein sequences

We obtained partial coat protein gene sequences for a total of 13 isolates of NNV from haddock, winter flounder and Atlantic cod (Fig. 3). Almost full length sequences of haddock NNV, Atlantic cod NNV from Nova Scotia, and Atlantic cod NNV from Newfounland have been deposited in Genbank® under the accession numbers AY547547-AY547549.

Comparison of the nucleotide sequence of a 925 bp region between isolates indicated a 99% nucleotide identity between the isolates obtained from Nova Scotia, New Brunswick and New Hampshire regardless of host species (Fig. 3). There were no differences in the deduced amino acid sequences between these isolates (not shown). Three Atlantic cod isolates from Newfoundland were sequenced and gave identical results. Comparison of NL isolates with the other isolates from this study indicated a 95% nucleotide identity and a 98% amino acid identity. These amounts to 5 amino acid substitutions within the T4 region of the Newfoundland isolate (not shown). Four of these substitutions resulted in the replacement of threonine with a non-polar amino acid. Differences within the Atlantic sequences appear to be related more to the geographical origin of the virus rather than to the host species from which it was isolated. The lack of deduced amino acid sequence differences amongst the isolates collected from hosts caught in New Brunswick, New Hampshire and Nova Scotia suggests that all of these isolates may be capable of infecting other hosts than the one that they were originally isolated from.

The closest sequence retrieved using BLAST similarity search on GenBank® was from Atlantic halibut NNV from Norway (accession no. AJ245641), with a nucleotide and amino acid identity of 93% and 96% respectively compared to the Atlantic cod isolates.

Phylogenetic tree inference

Analyses using the nucleotide sequences of the T4 region of the coat protein gene were conducted to infer the phylogenetic relationship of NNV isolates from the North Atlantic (Fig. 4). All the isolates reported in this study form a monophyletic group that we have referred to as the Atlantic cod NNV clade (ACNNV). Within the group, the isolate from Atlantic cod in Newfoundland (Cod02ac2) separates earlier suggesting that it is an ancestral form when compared to the rest of the group. The isolates from the Bay of Fundy area (region 3 on Fig. 1) are all closely related, and the isolate from New Hampshire is grouped with them, although their support value is somewhat low (Fig. 4). The Atlantic cod isolates from southern Nova Scotia are closely related, although they were recovered 3 years apart. One of the haddock isolates from the Bay of Fundy is grouped with the Atlantic cod isolates from Nova Scotia, but the grouping is not well supported.

The barfin flounder clade (BFNNV) is a sister group of the ACNNV (Fig. 4). Based on our phylogenetic analysis, the isolates within the ACNNV clade seem to have evolved for some time separately from the barfin flounder clade. We suggest that this level of difference as well as the monophyly of North Atlantic isolates and their clear separation from their sister clade (BFNNV) warrants the establishment of a new type species of NNV. We propose that

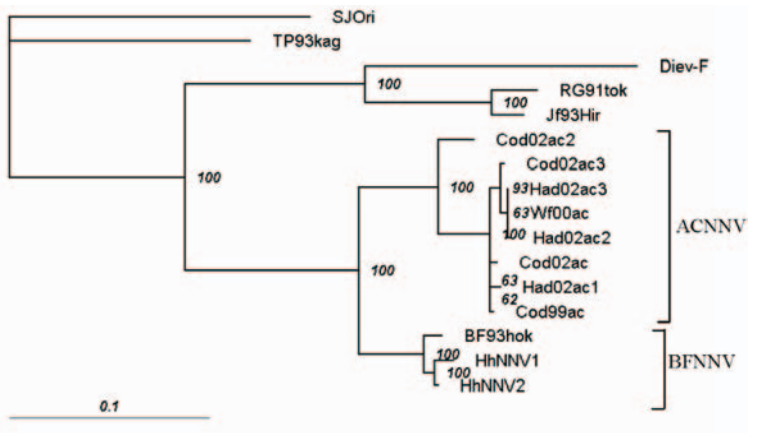


Figure 4
Unrooted consensus tree constructed with Tree-Puzzle 5.0 by the neighbour-joining method, and inferred with 1000 quartet puzzling steps and maximum likelihood branch lengths. The numbers give the quartet puzzling reliability (or support values) for the internal branches. The bar equals 0.1 nucleotide replacement.

this species be referred to as Atlantic cod NNV (ACNNV) as Atlantic cod was the first host species from which this virus was isolated.[6] Using an alphanodavirus (black beetle virus or BBV) as an outgroup indicated that the root of the inferred tree is located at a point that separates NNV isolated from cold water hosts (BFNNV and ACNNV) from NNV isolated from hosts in more temperate or tropical waters (not shown). Alternative inference methods were used to examine the phylogenetic relationship and these methods produced similar results (not shown).

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The Use of Molecular Biology in the Study of Host-Pathogen Relationships

Andrew Dacanay, Jessica M. Boyd, Laura L. Brown, Roger O. Ebanks, Stewart C. Johnson, Michael E. Reith, Kirty S. Solanky, and John A. Walter

Andrew Dacanay

Cutting edge molecular biology techniques, colloquially known as "biotechnology", are increasingly applied to the investigation of the fundamental biology of fishes, their pathogens and the mechanisms of infectious disease. Our institute routinely applies genomics, proteomics and metabonomics technologies to dissect the nature of the host-pathogen relationship between Atlantic salmon (*Salmo salar* L.) and one of its bacterial pathogens—*Aeromonas salmonicida*. Traditionally, determination of whether a gene or pathway was involved in virulence depended on whether a suitable mutant could be isolated after repeated passage; indeed many of the attenuated strains of *A. salmonicida* available today were generated that way. A genomics approach utilizing molecular tools such as bioinformatics, the PCR and cloning allows the creation of mutant strains precisely targeted to a specific gene. These mutants can then be used in traditional whole animal challenge trials, that when properly conducted, yield data on not only contribution to, but also mechanisms of, virulence. Knockout mutants require a target gene, and these can be identified experimentally as well as using bioinformatics tools. A proteomics approach, surveilling all the proteins produced by a cell (the proteome) can identify differentially expressed proteins which, when mutagenised, can also reveal a role in virulence. It is often a truism that genomics and transcriptomics, studies of genes and patterns of gene expression, do not coincide with proteomics as RNA and proteins have varying half-lives. Some researchers, therefore, advocate the use of metabonomics, a survey of metabolites, to be as revealing as genomics or proteomics. Metabonomic studies of *A. salmonicida* infected salmon reveal not only that they can be distinguished from uninfected controls but its quantitative nature allows physiological processes to be deduced. Metabonomics thus can be added to the powerful suite of biotechnology tools used in research programs focused on developing integrated health management strategies.

"This is an exciting time to be in fish health research—molecular tools are no longer the preserve of human or higher vertebrate veterinary medicine."

Introduction

Aeromonas salmonicida is a Gram-negative bacterium and is the aetiological agent of a bacterial septicaemia of teleosts, specifically known as furunculosis in salmonids. Many important processes of furunculosis are poorly understood, including its environmental reservoirs, route and mechanism of entry into the host and other virulence factors. The application of molecular biology, also known as

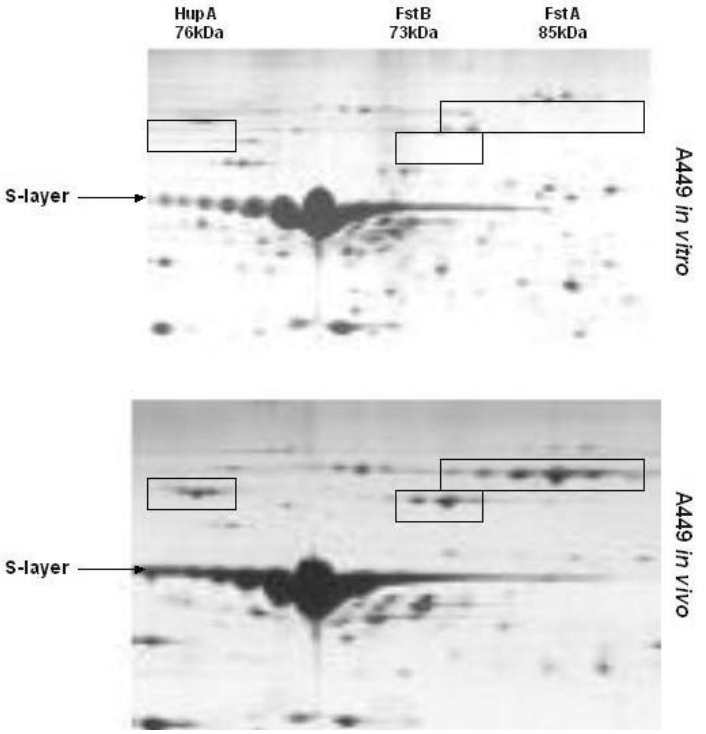

Figure 1
Detection of siderophore receptors FstA, FstB and HupA under standard *in vitro* growth in TSB (Top) and *in vivo* (Bottom) by 2 dimensional electrophoresis. All three proteins are repressed *in vitro* (Top) and expressed *in vivo* (Bottom, adapted from Ebanks.[5]

biotechnology, can help us understand these more fully. This will lead to a better understanding of furunculosis and reveal novel pathogen control strategies for this disease. It will also further our general knowledge of the host-pathogen relationship. This is significant as *A. salmonicida* is related closely to many important human pathogens including *Yersinia pestis, Vibrio cholerae* and *Escherichia coli.*

One of the strengths of molecular biology is that the investigator can precisely target, and hence study, a single gene or protein. Recent advances in DNA microarray technology mean that expression levels of thousands of genes can be monitored simultaneously. The precision inherent to molecular biology, however, means that undescribed genes or proteins may be intractable to further study. The conservation of key proteins and genes at the molecular level is such that gene identity or function in a poorly understood organism, such as *A. salmonicida*, can be inferred using data from more well understood organisms, such as *E. coli* or *Pseudomonas aeruginosa.* As recently as four years ago, little was known of *A. salmonicida* at the molecular level. There were only a handful of DNA sequences available in publicly accessible databases such as GenBank (www.ncbi.nlm.nih.gov/). Since this time the DNA sequencing unit at the National Research Council of Canada's Institute for Marine Biosciences (NRC-IMB, www.imb.nrc.ca) has a draft sequence of the ca. 4.6 Mbp (mega base-pair) *A. salmonicida* genome. Simultaneous data mining of the genomic sequences using bioinformatics tools has revealed upwards of 4000 genes. Of these, ca. 3400 have been identified by homology to genes in other bacteria. The challenge facing the NRC-IMB *Aeromonas* team is to confer biological relevance to these genes.

Identification of putative virulence factors

Of the 3400 or so genes identified in the *A. salmonicida* genome by homology to other species' genes, many were housekeeping genes that may have little or no effect on virulence. A small subset of genes had been implicated in virulence in *A. salmonicida* itself, such as a type IV pilin gene *tapA*[1] and the A- or S-layer gene *vapA*,[2, 3] whereas others (*sodB*) had been implicated in virulence of other bacteria.[4] This subset of genes, which will be discussed later, formed an interesting pool of genes available to study.

Other genes of interest were determined experimentally. Proteomics is the study of all expressed proteins in a cell or organism. This pool of expressed proteins will depend on the conditions under which the cell or organism finds itself. In one study [5] proteins up-regulated in response to the host were identified by comparing the *A. salmonicida* proteome when cultured under both standard laboratory conditions (tryptic soy broth, TSB) or under *in vivo* conditions using a surgically implanted bacterial growth chamber by 2-dimensional gel electrophoresis (2-DE,

Fig. 1). Using mass spectroscopy (MS), we were able to obtain short amino acid sequences for these proteins, which were then identified as siderophore receptors using the *A. salmonicida* database. Siderophore receptors are integral membrane proteins involved in iron uptake and considered important virulence factors in other species of bacteria including *Yersinia enterocolitica*[6] and *Vibrio* (now *Listonella) anguillarum*.[7] Two siderophores had been identified previously in *A. salmonicida* by Hirst and Ellis[8] using SDS-PAGE. In this instance the additional precision offered by 2-DE allowed us to identify an additional up-regulated protein as well as permitting MS-based sequencing to identify it unambiguously. Other studies identified manganese co-factored superoxide dismutase, sodA, as being up-regulated under *in vivo* conditions.[9,10] *sodA* is involved in the detoxification of toxic oxygen free radicals that are both unwanted byproducts of respiration and produced as an antimicrobial by host immune cells such as macrophages. These proteins were added to the list of putative virulence factors identified in silico, as being suitable for gene knock-out studies.

Gene Knock-out Mutants

In knockout mutants, a gene of interest is identified *in vivo* or in silico as above. A short fragment of that gene is amplified by the polymerase chain reaction (PCR) and cloned into a circular piece of extra-chromosomal DNA called a plasmid. When the plasmid is introduced into a bacterial cell, the plasmid integrates into the gene of interest on the bacterial chromosome by homologous recombination. Specificity for the gene of interest is provided by the cloned PCR fragment. The gene is now unable to function as it contains several thousand (kilo base-pairs) of extra DNA that disrupt transcription. Whereas knockouts in *A. salmonicida* have been generated before,[11,12] the NRC-IMB *Aeromonas* team has systematically produced knockout strains in several pathways suspected to be important in the virulence of *A. salmonicida*.

Once constructed, the mutant strain can be studied to determine the effect of knocking out the chosen gene. In the terms of the host-pathogen relationship, the effect of most interest is the virulence of the mutant strain: the ability, or inability, to cause disease. This is usually determined by challenge of a suitable host with the knockout, using the parental strain as a control. The data from several challenge trials conducted at NRC-IMB are summarized in Table 1. *tapA* and *flpA* are type IV pili genes. Pili are short, filamentous extracellular structures that are required for adherence and invasion of the host and are considered amongst the most important virulence factors in *P. aeruginosa*.[13] These genes were identified in silico in the *A. salmonicida* genome. The siderophore receptor genes *fstA*, *fstB* and *hupA* and a superoxide dismutase (*sodA*) were identified experimentally *in vivo*.[5,9,10]

Challenge

There are various methods for challenge trials, each with its own advantages and disadvantages.[14] We routinely conduct challenge trials by immersion, a more natural route of exposure than an intraperitoneal (i.p.) injection, and one which allows us more control over the infection process than a cohabitation challenge. As can be seen in Table 1, these knockouts all had effects on virulence, with the exception of FstB. Knocking out *sodA* (*sodA*$^-$) had a modest effect on virulence, increasing relative percent survival (RPS) and mean time to death (MTTD), a measure of the length of the experimentally induced infection. Similarly, two of the three siderophore receptor knockouts either increased RPS, increased the

mean time to death, or both. The results of the challenges with *tapA*⁻ and *flpA*⁻ as well as a *tapA*⁻/*flpA*⁻ double knockout, are instructive as to why flexibility must be retained when designing challenge trials. By i.p. injection there were no significant differences in mortality or MTTD in either single or the double mutant and the wild type. By immersion, mortality was consistently lower than wild-type in both *tapA*⁻, *flpA*⁻ and *tapA*⁻/*flpA*⁻, significantly so for both *tapA*⁻ and *tapA*⁻/*flpA*⁻. These data suggest that both pili operons are important to virulence, *tapA* more than *flpA*. This was only apparent by immersion challenge, not by i.p. injection. This confirms that the pili are only important for virulence if the bacteria are required to adhere to, and invade, the host. If the need for adherence and invasion is abolished by introducing the bacteria directly into the peritoneal cavity, pili are essentially superfluous and knocking out either *tapA* or *flpA* had no measurable effect on virulence.

Metabonomics

Metabonomics provides biochemical information at the metabolite level. Proton Nuclear Magnetic Resonance (^{1}H NMR) is used to survey small metabolites in body fluids such as plasma, urine or cerebrospinal fluid.[15-17] Plasma can be repeatedly and non-lethally sampled from fishes, making it an ideal biofluid for time-based metabonomic studies of the teleost host in response to the pathogen. Blood was drawn from animals that had survived some of the challenge trials outlined above and subjected to ^{1}H NMR. The resulting spectra were further analysed by Principle Components Analysis (PCA). In PCA each spectrum is assigned to a position in k-dimensional space based on its spectral characteristics.[18] In studies of laboratory rodents and even humans, spectra from individuals under similar conditions tend to cluster. This has led to the development of metabonomic analysis as a rapid, relatively non-invasive technique for use in human diagnostics.[17] We were interested to see if we could apply the same technique to aquatic animal disease. The data suggest that we can. PCA analysis of plasma from the two groups showed that they clustered separately[19] (Fig. 2). NMR is quantitative as well as analytical; analysis of individual spectra not only identifies the metabolites that have changed but can quantify them also. Further interrogation of the spectra revealed that the major metabolites that contributed to this clustering were glucose, various amino acids and lipid/protein complexes, a profile consistent with starvation. Anorexia is a commonly reported sign of furunculosis so we withheld feed from another control group for ten days before performing a similar analysis. The experimen-

Table 1. Survival statistics from challenge trials with knock-out mutant strains of *Aeromonas salmonicida*. Knock-out genes are: *sodA*—manganese cofactored superoxide dismutase; *fstB, fstA, hupA*—siderophore receptors; *tapA, flpA*—type IV pilin subunits. RPS, relative percent survival calculated as as (1-(test mortality/control mortality)) x 100. MTTD , mean time to death, is the time in days to reach 50% of the total recorded cumulative mortality. N/D, not determined. Challenge exposure route: dip, immersion; i/p, intraperitoneal injection. RPS is negative for the *flpA*⁻ i/p challenge as a 10x inoculum of this knockout was administered inadvertently.

Strain	Route	RPS	MTTD/days
Wild-type	dip	N/D	6
sodA⁻	"	35.6%	13
fsA⁻	"	5.0%	N/D
fsB⁻	"	97.2%	15
hupA⁻	"	33.3%	8
tapA⁻	"	35.4%	13
flpA⁻	"	26.7%	17
flpA⁻/*tapA*⁻	"	14.4%	14
Wild-type	i/p	N/D	6
tapA⁻	"	13.3%	7
flpA⁻	"	-11.1%	7
flpA⁻/*tapA*⁻	"	11.7%	8

tally starved animals formed a third cluster that occupied some of the same spectral space as the original control group but did not overlap with that of the challenged animals. Therefore, metabonomics can distinguish challenged animals from uninfected animals. An unexpected finding was that two of the nine challenged animals that were analysed clustered with the controls. Of the surviving challenged animals we would expect to find that up to 80 or 90% of them would have a clinically inapparent, or covert, infection. Even though the disease state of these animals was not defined by a stress test, we speculate that these two (22%), apparently anomalous, spectra represent this small, non-covertly infected sub-population.

Summary

This is an exciting time to be in fish health research—molecular tools are no longer the preserve of human or higher vertebrate veterinary medicine. Yet, biotechnology is not gratuitous reduc- tionism. These tools have enabled us to dissect further the nature of the host-pathogen relationship between *A. salmonicida* and *S. salar* and it has allowed us to explore new avenues for non-lethal diagnosis and fish health management. These tools surpass the skill-set of a single individual or even individual laboratory; a comprehensive research program, such as this, includes the integration of microbiology, fish-health, immunology, molecular biology, biochemistry, computing (bioinformatics) and physics laboratories, at which point their output is synergistically worth more than the sum of their parts.

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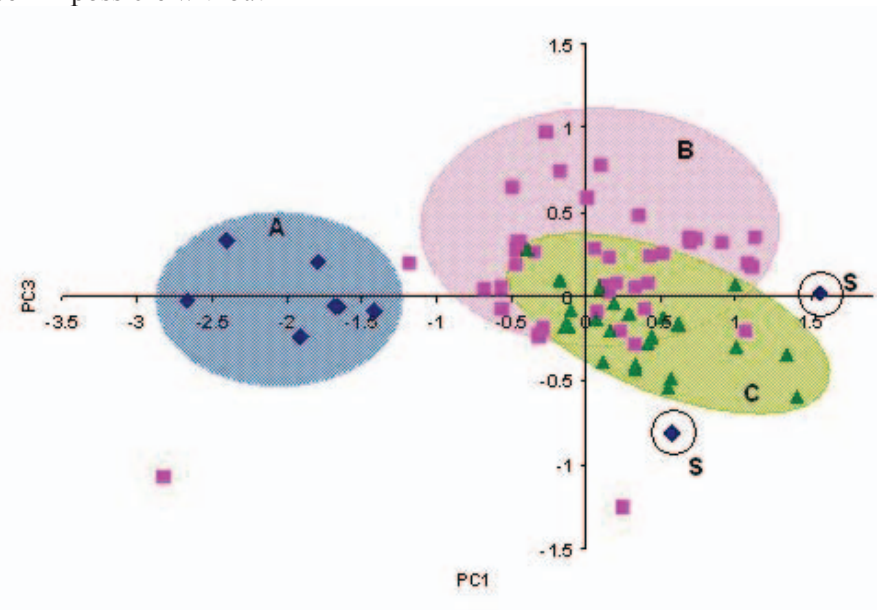

Figure 2. Principal Components Analysis of ^{1}H NMR spectra from Atlantic salmon plasma from fish that survived an *A. salmonicida* challenge (A), naïve controls (B) and starved naïve controls (C). Spectra from the challenged and control fish cluster separately. The spectra from the starved fish clustered with the control fish, suggesting that the differences between challenged and unchallenged salmon were not entirely related to the metabolic effects of anorexia, which is a common clinical sign of *A. salmonicida* infection, rather they were due to the disease process itself. Two challenged fish (circled, marked "S") clustered with the controls suggesting that they were not infected. As all fish were outwardly healthy at the time of sampling this further suggested that the challenged cluster may also represent animals with a clinically inapparent, or covert, infection. Adapted from Solanky et al.[19]

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Molecular Studies on Infectious Salmon Anaemia Virus (ISAV)

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Infectious salmon anaemia (ISA) virus (ISAV) is currently one of the most important viral pathogens threatening commercial aquaculture in the northern hemisphere. The virus is classified in the family *Orthomyxoviridae*, genus *Isavirus*. The virus offers a significant challenge to fish biologists interested in diagnosis and control of ISA, and to molecular virologists because of its evolutionary relationship with influenza viruses. Being an orthomyxovirus, ISAV is expected to be prone to genetic and antigenic variation. However, while the ISAV genome, like the influenza A virus genome, encodes at least nine structural proteins, the gene order and putative functional identities of the ISAV proteins are significantly different from those of influenza viruses. Although the virulence of influenza A viruses is a polygenic trait, one virulence factor is correlated with the haemagglutinin cleavage site. It remains to be shown which genetic variations in ISAV are associated with the virulence of the virus. The ability of ISAV to kill rainbow trout is a correlate of ISAV pathogenicity that might facilitate the identification of ISAV virulence genes.

Introduction

Infectious salmon anaemia (ISA) virus (ISAV*)* is a segmented, negative-sense, single-stranded RNA virus[1,2] of the family *Orthomyxoviridae,*[3] the only member in the genus *Isavirus* (http://www.ncbi.nim.nih.gov/ICTBdb/lctv/index.htm, accessed January 15, 2004). ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon (reviewed in Kibenge et al. [4]) and asymptomatic infection in wild fish.[5] The clinical ISA disease can also occur in wild free-ranging Atlantic salmon, but these fish are less susceptible than the farmed Atlantic salmon.[6] In this communication, I will briefly review what is currently known about the structure of ISAV and how it might relate to pathogenic differences among ISAV isolates.

Several theories have been put forward to explain the geographic and host origin, and therefore the virulence, of ISAV in farmed fish.[6-10] For example, it was suggested that ISAV may have been introduced to Norway with the importation of rainbow trout from North America.[11] It has been suggested that there are natural reservoirs for the virus, probably in fish occurring in the coastal areas where ISA outbreaks frequently occur.[12] Thus the virulence of ISAV is intrinsically linked to its emergence as a pathogen in marine farmed Atlantic salmon.[8] However, the correlates of pathogenicity of ISAV are as yet poorly understood. It is assumed but not yet proven that there is variation in pathogenicity among ISAV strains. Because only samples from clinical cases routinely receive appropriate diagnostic attention, naturally avirulent ISAV strains may be rarely isolated, if at all. It has not been possible to isolate virus from some natural ISA outbreaks[8] and from

some ISAV RT-PCR-positive fish,[13] probably because the available fish cell lines are not sensitive enough to grow low virus titres (for example, the available fish cell lines may not be as sensitive for replication of pathogenic ISAV as are naïve Atlantic salmon) and are not permissive to non-pathogenic ISAV strains.

From our research work, it is apparent that a combination of virus virulence and host susceptibility determines the outcome of ISAV infection in fish. Thus a highly virulent virus in a resistant host such as rainbow trout will have a similar outcome as a virus of low pathogenicity in a highly susceptible host such as Atlantic salmon.[4] The highly pathogenic strains of ISAV are also more aggressive in cell culture[4] indicating that a key attribute of ISAV virulence is the rate of virus replication. The rate of virus replication is a recognized indicator of virulence in influenza A viruses, which in chickens and turkeys group into one of two extremes of virulence, highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses.[14]

The rate of virus replication in avian influenza viruses is correlated with the haemagglutinin cleavage site. The LPAI viruses which have only two basic amino acids at the cleavage site are only cleaved by trypsin-like enzymes and are thus restricted to replication at sites in the host where such enzymes are found, i.e., respiratory and intestinal tracts. On the other hand, the HPAI viruses have multiple basic amino acids at the cleavage site,[15] allowing the haemagglutinin precursor to be cleavable by ubiquitous host proteases.[16] Consequently, the HPAI viruses are able to replicate systemically with resultant multiple organ and tissue damage with severe disease and death.[17] It has been demonstrated that the HPAI viruses do not constitute a separate phylogenetic lineage or lineages but appear to arise from low pathogenic precursor strains.[14,18,19] By analogy, the ISA disease in Atlantic salmon is also caused by novel virulent strains of ISAV that emerge from background benign infections in wild fish and then adapt to the new host, possibly through haemagglutinin gene deletions[10] during intensive aquacultural practices.[20,21] However, there is no cleavage of the ISAV haemagglutinin protein,[22] and no marker for virulence has yet been definitively associated with this gene in ISAV.

The ISAV haemagglutinin protein contains a hypervariable region[22-24] with a highly polymorphic region (HPR) which shows temporal and geographical sequence variation.[9] A total of 11 HPR groups (1-11) were identified among 37 ISAV isolates, with HPR1 and HPR2 having been present prior to 1993, and the later isolates possibly arising by deletions within the HPR.[8,9] Mjaaland *et al*.,[10] on the other hand, analyzed 76 isolates and came up with a different set of 19 polymorphic region (PR) groups (0-18), albeit encompassing the signature motifs of HPR1 and HP2 groups of Devold *et al*. [9] These authors were of the view that the patterns of variability in the PR were the result of deletions from a longer ancestral haemagglutinin sequence designated PR-0. The PR-0 sequence was amplified from healthy wild Atlantic salmon in Scotland[25] and has been hypothesized to be the source of virulent ISAV isolates that contain various deletions in the haemagglutinin gene, now separated into 16 HPR groups (0-13, 20 and 21).[8] Most of these HPR groups are represented by isolates with the European haemagglutinin genotype. It was suggested by Mjaaland *et al.* [10] that the more uniform sequence patterns of the North American haemagglutinin genotype may be indicative of these isolates having a single, more recent common source. Mjaaland *et al.*[10] attempted to correlate the polymorphism in the haemagglutinin protein with ISAV virulence by grouping ISA outbreaks into acute and protracted forms. It was observed that virus could be isolated from all samples from acute ISA outbreaks, and from 4 of 9 protracted ISA outbreaks, but from none of the

samples from farms without clinical disease. However, there was no clear correlation with the PR groups or with the virus replication rate of the isolates from acute and protracted ISA outbreaks in SHK-1 cells.[10]

On the basis of our recent studies, we can separate ISAV isolates into those that kill rainbow trout and those that do not, and this separation appears to correlate with ISAV pathogenicity in Atlantic salmon, and with the rate of virus replication in several cell lines. Thus the rainbow trout infection phenotype might facilitate the identification of ISAV virulence genes.

". . . we can separate ISAV isolates into those that kill rainbow trout and those that do not, and this separation appears to correlate with ISAV pathogenicity in Atlantic salmon, and with the rate of virus replication in several cell lines."

Structure of ISAV

ISAV shares several morphological, biochemical, and physicochemical features with those of influenza viruses. It is the only species of the genus Isavirus, one of the five genera of the family *Orthomyxoviridae*. Viruses in the genus *Isavirus* are enveloped particles of 90-140 nm diameter[6,26-28] with 13-15 nm long mushroom-shaped surface projections consisting of a combined receptor-binding haemagglutinin and receptor destroying enzyme activity demonstrated to be an esterase, hence recently designated HE.[29] The genome is composed of eight segments of linear, single-stranded negative sense RNA ranging in length from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb.[30] All the eight RNA segments of ISAV have been sequenced.[2,3,22,23,30-34] Comparison of the ISAV proteins with those of other orthomyxoviruses revealed low amino acid identity values, between < 13% and < 25%,[3,24,32,33] and the order of the genome segments encoding the proteins in ISAV appears to differ from those of influenza viruses.[4]

The structural protein profile of ISAV has not been conclusively determined,[1,13,30,35] as the identities of the proteins encoded by most of the segments have not been documented (Fig. 1). Only the putative PB2, haemagglutinin, and segments 7 and 8 genes have been expressed in heterologous systems although none of these proteins have been purified to homogeneity. For the ISAV putative PB2 protein, functional analysis using the segment 1-EGFP fusion construct and transfection in CHSE-214 cells revealed nuclear localization of the protein.[34] The ISAV haemagglutinin gene was expressed both in a baculovirus system with Sf9 cells[22] and by using the segment 6-EGFP fusion construct and transfection in SHK-1 cells.[23] The protein which was expressed either in Sf9 cells or SHK-1 cells was localized exclusively in the cytoplasm, it was recognized by monoclonal antibody 3H6F8 which is directed against the ISAV haemagglutinin,[36] and it caused the cells to bind salmon erythrocytes. No acetylesterase activity was observed in the transfected SHK-1 cells,[23] leading the authors to suggest that the haemagglutinating and acetylesterase activities were carried out by two different proteins in ISAV. However, most recently, it was demonstrated by labeling of purified ISAV with [1,3-3H]diisopropyl fluorophosphate that the viral esterase is located with the haemagglutinin in the gp42 protein, hence the proposal to call it haemagglutinin-esterase (HE) protein.[29] The heterologously expressed protein had a predicted molecular mass of 42.4 kDa similar to that demonstrated by immunoblot analysis, indicating that the ISAV haemagglutinin, in contrast to influenza A virus HA, is not post-transcriptionally cleaved.[22] Biering et al.[37] expressed segment 7 open reading frame (ORF) 1 and the larger ORF of segment 8 in *Escherichia coli* and pre-

Figure 1
The structural profile of the ISAV particle has not been conclusively determined.

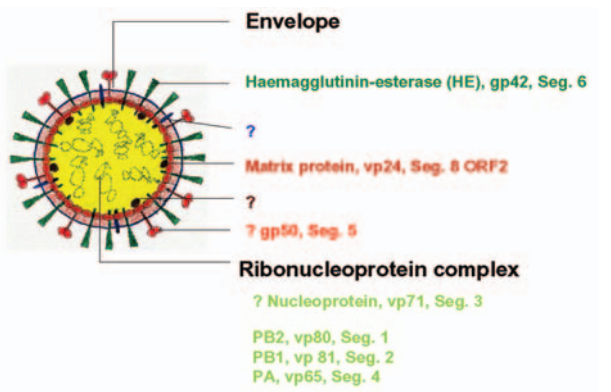

Table 1. Comparative virulence of infectious salmon anaemia virus isolates (data are sumarized from Kibenge et al., in prep.)

	Percent mortality in different fishes[a]			Virus titre on different per missive cell lines		
ISAV Isolate	Atlantic salmon	Rainbow trout	Coho salmon	CHSE-214	ASK-2	TO
NBISA01	95.6 (10-19)[b]	50 (13-27)	0	5.16[c]	7.16	8.16
RPC/NB 980-49-1	100 (11-23)	10 (18)	0	3.83	ND	7.83
810/0/99	100 (13-33)	20 (30-40)	0	< 1.0*	6.50	8.16
390/98	79.2 (11-23)	0	0	—	4.50*	6.50*
RPC/NB 98-0280-2	66.7 (11-28)	10 (33)	0	—	5.83	7.83
U5575-1	64.0 (18-39)	0	0	—	5.16*	6.83*
485/9/97	60.0 (18-41)	0	0	< 1.0*	4.17*	ND
RPC/NB 00-0593-1	50.00 (15-30)	0	0	< 1.0*	5.50	7.83

[a] Fish weighing approx. 20 g were given $10^{5.80}$ $TCID_{50}$/0.2mL/fish intraperotoneally of virus grown in TO cell line (except NBISA01 which was grown in CHSe-214 cell line).

[b] Numbers in parentheses refer to duration of mortality in days post-inoculation.

[c] Virus titres are expressed as $TCID_{50}$/mL; — denotes no CPE onserved; * indicates slow developing (incomplete/limited CPE in cell monolayer; ND denotes not done.

pared rabbit antibodies to the expressed proteins. Then by immunoblot analysis using native and expressed proteins and rabbit antibodies to whole virus and expressed protein, they demonstrated an association between the 24-kDa major structural protein[1] and the segment 8 product.[37] This protein corresponds to the vp22 protein in lysed virions that was recently shown to be a late protein accumulating in the nucleus, and designated the matrix (M) protein.[29] Because the segment 7 ORF1 protein did not react with ISAV antiserum, it was considered to be nonstructural or a minor structural protein.[37] We studied the gene-coding assignments of ISAV by individually expressing the ORFs in the eight RNA segments in vitro with rabbit reticulocyte lysates and analyzing their translation products by immunoprecipitation with rabbit antiserum to purified whole virus.[4] Our data shows that the ISAV genome encodes at least 10 proteins, of which at least nine are structural and one is nonstructural. Some of these proteins have unique motifs, for example the putative nucleoprotein (NP) has a transmembrane motif, which is unusual for a nucleocapsid protein, and needs further study.

Rainbow Trout Infection Phenotype of ISAV

In an attempt to identify and characterize the correlates of pathogenicity of ISAV, we compared the infectivity of different ISAV isolates in three different farmed fishes (Atlantic salmon, coho salmon, and rainbow trout) to the extent of virus replication in three permissive fish cell lines (TO, ASK-2, and CHSE-214). Gene sequence variations of the different ISAV isolates were also analyzed in search of ISAV virulence markers (Kibenge et al., in prep.). The ISAV isolates used varied in their mortality patterns for Atlantic salmon (Table 1) reflecting a variation in their virulence. Thus the most virulent virus strains, NBISA01, RPC/NB 98-049-1, and Norway 810/9/99, had the highest mortality of >90%. Fish infected with these viruses had the most acute mortality phase in Atlantic salmon, starting between 10 and 13 days post infection (dpi) and lasting for only

9 to 15 days. The Atlantic salmon mortality of the lesser virulent ISAV isolates either lasted longer and/or started later portraying a protracted mortality phase. For example, in isolates U5575-1 and Norway 487/9/97 with mortalities of 64% and 60%, respectively, the first mortality occurred at 18 dpi and the last at 39 and 42 dpi, respectively. When the ISAV isolates were ranked based on ability to kill Atlantic salmon, the isolates with the highest cumulative per cent mortalities (i.e., >90%) and some with medium cumulative per cent mortalities (65-85%) also killed rainbow trout (Table 1). The cumulative per cent mortality in rainbow trout was always lower than in Atlantic salmon. In addition, the mortality phase in ISAV-infected rainbow trout was chronic in that it either started later and/or lasted longer than in Atlantic salmon for the same virus isolates. On the basis of these data, the in vivo correlates of virulence of ISAV include the following: 1) Cumulative per cent mortality in Atlantic salmon; the most pathogenic strains had mortality levels of > 90%, 2) Nature of mortality phase (i.e., time of onset and duration of mortality) in Atlantic salmon; the most pathogenic strains had an acute mortality phase that started at 10-13 dpi and lasted for 9-15 days, and 3) Mortality in rainbow trout, with systemic haemorrhagic lesions and an in situ hybridization pattern with ISAV riboprobes that were consistent with those typically seen in Atlantic salmon infected with ISAV. The highly pathogenic strains were also more aggressive in several cell lines, inducing CPE sooner and more completely in most permissive cell lines than the less pathogenic ISAV isolates. Analysis of natural ISA disease patterns has also suggested a grouping of two main forms, acute and protracted.[10] Therefore, we can separate ISAV isolates into those that kill rainbow trout and those that do not, and this separation appears to correlate with ISAV pathogenicity in Atlantic salmon, and with the rate of virus replication in several cell lines. Thus the rainbow trout infection phenotype might facilitate the identification of ISAV virulence genes.

Correlation of ISAV Virulence with *N*-Glycosylation In HE Protein

More nucleotide sequence data are available on the HE gene (RNA segment 6) than on any other gene of ISAV (GenBank database) because of the assumed role of the viral haemagglutinin protein for recognition and attachment to host cell surface receptors terminating in sialic acid, which is a pre-requisite for virus entry into the cell during virus infection. Moreover, the same protein in ISAV is known to possess the receptor destroying enzyme activity.[29,38] Previously, the variation in the size of this protein among ISAV isolates from New Brunswick, Canada, was linked to cell-line permissiveness,[39] indicating that genetic variation in ISAV segment 6 could be involved in the differences in natural ISA outbreaks and cell culture replication properties. Indeed Mjaaland *et al*.[10] correlated the 19 PR groups with disease development and virus replication in SHK-1 cells. The HPR of the HE protein includes two predicted *N*-glycosylation sites (^{333}NIT^{335} and ^{349}NQT^{351}) that would lie in the "stem" of the HE protein, immediately outside the viral envelope.[10] Recently, we were able to obtain virus neutralization data that correlated with the presence of the putative antigenic motif in the HE protein around the common potential *N*-glycosylation site at amino acid positions ^{333}NIT^{335}.[4] We speculated that the second unique potential *N*-glycosylation site at amino acid positions ^{349}NQT^{351} closest to the predicted transmembrane region, in the Nova Scotia ISAV isolate U5575-1 HE protein (Fig. 2) may result in a new epitope, accounting for this isolate and Norwegian isolate 485/9/97 being antigenically distinct from the European haemagglutinin subtype.[4] Isolate U5575-1 belongs to the HPR3 group of Devold *et al*. [9] and Nylund *et al*. [8] and

PR3 group of Mjaaland *et al*.,[10] but the additional glycosylation site is also present in isolates belonging to HPR groups 0, 2, 9, and 12,[8,9] and PR groups 0, 1, 2, and 4,[10] indicating that this serotype is common among ISAV isolates.

Because isolate U5575-1 is also of lower pathogenicity (Table 1), it is hypothesized that the additional glycosylation site at amino acid positions ^{349}NQT^{351} also contributes to its reduced virulence, i.e., that a gain of *N*-linked glycosylation in the HE protein alters virulence of ISAV. This hypothesis is supported by the following observations by others[8-10]: 1) the additional glycosylation site is present in the HPR0 ancestor, and 2) among isolates from all areas where ISA outbreaks have been reported [New Brunswick-AF427045; Nova Scotia-AF294881; PR1-4 groups from Norway;[10] Scotland-AF391126; Faeroe Islands-isolate F72/02;[8] and possibly Maine, USA-AY575955]; and 3) it is not present in the earliest ISAV isolate ever sequenced on segment 6 [Norway-AF364893] which could have allowed its collection back then, and subsequent isolation in cell culture. Glycosylation of the haemagglutinin protein is known to be essential for proper folding of the protein in influenza A virus[40,41] and for virus replication in mammalian cells.[42] In Newcastle disease virus (NDV) where the haemagglutinin-neuraminidase (HN) protein is an important determinant of virulence, a loss of *N*-linked glycosylation from the HN protein resulted in loss of virulence of NDV.[43] Other ISAV RNA segments that have been sequenced for several isolates including the Nova Scotia virus U5575-1 seem to separate the isolates along geographical sources of origin rather than pathogenicity.

Conclusions

In conclusion, ISAV isolates that kill rainbow trout are highly pathogenic for Atlantic salmon. This rainbow trout infection phenotype might facilitate the identi-

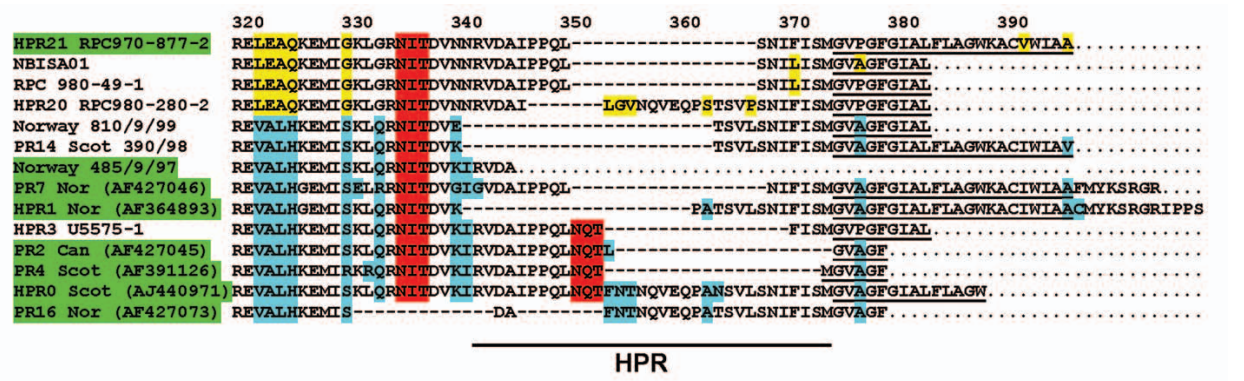

Figure 2

Molecular basis for ISAV serotypes (and virulence?). Alignment of deduced amino acid sequences of hypervariable region of haemagglutinin-esterase (HE) protein of selected ISAV isolates identifies a putative antigenic motif around the common potential *N*-glycosylation site at amino acid positions ^{333}NIT^{335}. Dashes indicate deletions while dots denote unsequenced regions in the respective virus isolates. The transmembrane region in each sequence is underlined. The extent of the highly polymorphic region (HPR)[9] is also indicated.

An additional potential *N*-glycosylation site at amino acid positions ^{349}NIT^{351} in ISAV isolate U5575-1 may account for this isolate [& possibly Norwegian isolate 485/9/97, and by implication isolates belonging to HPR groups 2, 3, 9, and 12 of Devold *et al.* [9] and Nylund *et al*., [8] and PR groups 0, 1, 2, 3, and 4 of Mjaaland *et al*.[10]] being antigenically distinct from the European haemagglutinin subtype [and of reduced virulence?]. Isolates in green were not used in challenge experiments (Table 1) and are included here for reference only.

fication of ISAV virulence genes. While sequences for most ISAV genes seem to separate the isolates along geographical sources of origin, the HE protein (RNA segment 6) sequence would seem to group isolates into three antigenic types (serotypes); the Nova Scotia ISAV isolate U5575-1 has an additional unique potential *N*-glycosylation site at amino acid positions ^{349}NQT^{351} that accounts for this isolate being antigenically distinct from the European haemagglutinin subtype. Because isolate U5575-1 is also of lower pathogenicity, and exhibits limited ability to replicate in cell culture, it is hypothesized that a gain of *N*-linked glycosylation in the HE protein alters virulence of ISAV. Challenge fish experiments need to be performed with more ISAV isolates having this additional *N*-glycosylation site to further test this hypothesis.

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Molecular Tools for Aquatic Animal Health: Past and Future Perspectives

Douglas McIntosh

The use of molecular tools for both diagnosis and prevention of aquatic animal diseases has become increasing popular during the last 10 years. Despite some initial resistance, advances in the fields of human and veterinary molecular biology and microbiology have been embraced, applied and adapted to the study of aquatic animal health. A bewildering array of molecular tools is now available and the development of new methodologies is continuing at a rapid pace. The need to have ever more rapid and sensitive methods for diagnosis of disease has been met, in part, through the wide scale utilization of polymerase chain reaction (PCR) based assays. However, it would be fair to say that thus far molecular detection has been used primarily as a research tool rather than as the "Gold Standard" method for pathogen detection. Confidence in the reliability of molecular methods is growing globally among the regulatory agencies responsible for aquatic animal health and it seems certain that in the future more and more diagnoses will employ PCR-based methods.

In the field of disease control, molecular methods have been used on two main fronts. Firstly, for the development of recombinant vaccines and secondly for the molecular typing of pathogens. Typing methods used to date include pulsed field gel electrophoresis, ribotyping and a variety of PCR-based techniques. Once again, the use of these methodologies will continue to expand to address problems which cannot be resolved using conventional approaches.

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Introduction

In common with terrestrial animal production, the issue of disease management is a priority in the culture of aquatic animals. Traditionally, the diagnosis of most microbial diseases of aquatic animals has been conducted using a relatively narrow range of diagnostic methods. In particular, histopathological or microbiological identification methods based on culture of the causative agent on agar plates or in cell culture have become the "Gold Standards", often in conjunction with confirmatory immunological techniques. However, the last 20 years have seen an explosive expansion of the field of molecular biology which has resulted in the development of a variety of tools for pathogen detection, detailed pathogen characterization/typing and vaccine development.

Pathogen Detection

The molecular tool which has the greatest potential to impact upon the detection of aquatic animal pathogens is the polymerase chain reaction (PCR). This technol-

ogy involves the amplification of pathogen-specific nucleic acid sequences present in fish tissues or environmental samples and does not require culture of the pathogen. A well designed PCR-based assay will demonstrate a high degree of specificity and sensitivity, allowing detection of the target pathogen even when present at low levels. Moreover, pathogen detection by PCR can be accomplished in hours rather than the days or weeks required for histological and routine culturing methods. Finally, PCR assays may be multiplexed, which provides them with the ability to check for the presence of several pathogens simultaneously. This feature is particularly useful when dealing with diseases that demonstrate very similar outward pathologies and which defy diagnosis by simple histological examination. PCR-based assays have been reported in the scientific literature for virtually all the major and many minor pathogens of economically important aquatic animals. Thus, the adoption of PCR-based diagnostic assays to replace or enhance more traditional methods appears to be an attractive option. It may therefore be somewhat surprising to discover that PCR-based detection methods are not widely employed as a primary or even secondary diagnostic method for any disease of aquatic animals. A notable exception is the use of reverse transcriptase PCR (RT-PCR), for the diagnosis of infectious salmonid anemia (ISA) in Atlantic salmon in Atlantic Canada and the USA. Although even here, a positive diagnosis by PCR must be substantiated by a positive result for either immunological or cell culture based detection of the virus.

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As noted above, a plethora of PCR assays have been developed and evaluated under laboratory conditions. Yet few of these have undergone detailed validation in field settings, which has raised doubts concerning the interpretation of results generated by PCR. This issue is particularly problematic for regulatory agencies responsible for implementing aquatic animal health management strategies. Thus, in order for PCR to attain full acceptance as a diagnostic tool it will be necessary for more rigorous validation studies to be undertaken.[(1)] At the same time it is worth noting that PCR can only provide evidence as to the presence of nucleic acid originating from a pathogen, it cannot provide information as to the viability of the pathogen and in the case of bacterial pathogens it provides no insight into antimicrobial sensitivity and therefore cannot aid in deciding upon treatment options. In conclusion, PCR will continue to offer an attractive alternative diagnostic method, particularly for conditions that require long, complex culture or histology-based confirmatory diagnosis. The issue of assay validation will require substantial investment, however. Once the reliability of individual PCR assays have been established, regulatory agencies should feel confident enough to introduce these techniques into surveillance programs and aquatic animal health will benefit as a result.

Pathogen Characterization

The characterization of aquatic animal pathogens at the species level represents an over simplification of many disease conditions including vibriosis and ISA. For this reason, the use of molecular methods capable of generating detailed information on the genotype of individual pathogens recovered from discrete disease episodes has become an increasingly popular subject in the field of aquatic animal health. At the simplest level, direct examination of the pathogen genome employing PCR assays using multiple sets of sequence specific primers designed to detect virulence genes or antibiotic resistance determinants, can provided valuable information concerning the occurrence of a particular variant of a pathogen. This approach has recently been used in our laboratory for the molecular characterization

of a multiple drug resistant strain of *Aeromonas salmonicida* responsible for significant salmon smolt mortalities in New Brunswick in 2003. These data helped confirm the spread of this variant between different sites and will be used to screen any new outbreaks which may occur.

For those wishing a more detailed dissection of their pathogens, the "molecular tool box" offers a continually expanding array of methods which allow the discrimination of microbial pathogens. These methods include pulsed field gel electrophoresis (PFGE), plasmid profiling, arbitrarily primed PCR, PCR-DNA sequencing and polyacrylamide gel electrophoresis for analysis of proteins, lipopolysaccharide and glycoproteins. The principles and applications of these methods have been reviewed recently.[2] Arbitrary PCR-based typing methods are among the most commonly employed techniques in the study of pathogens of aquatic animals. In contrast to conventional PCR, these techniques involve the use of primer sets which have been designed to bind at numerous, randomly distributed sites throughout the genome. The amplification reaction results in the production of numerous products of different molecular mass. Subsequent separation of these products by electrophoresis on agarose or acrylamide gels, yields a pattern of bands which can often be unique for a particular isolate.[2] These patterns, sometimes referred to as "pathogen barcodes", can be stored digitally using powerful image processing software allowing their subsequent retrieval for comparison with new isolates processed and typed using the same methods, even when the typing was performed on the other side of the world. In general, typing studies are retrospective and involve the examination of large numbers of isolates or libraries produced from a variety of outbreaks, often collected over months or even years. The extreme discriminatory power of these molecular typing methods allows a detailed examination of the frequency and distribution of a given pathogen, thus providing a solid base from which to assess the magnitude and impact of a given disease problem at regional, national and international levels. The continued application of these methods to the molecular characterization of pathogens seems certain. In common with PCR validation for diagnostic purposes, the development of pathogen databases will require significant investment. Yet, it is obvious that as aquaculture expands to encompass an ever growing number of animal species, new pathogens or variants of old pathogens will emerge and that they will need to be classified to allow the successful implementation of control and treatment strategies.

"Molecular methods have also found a substantial role in the development of novel vaccines based upon recombinant DNA technology."

Vaccine Development

Molecular typing methods impact upon vaccine development for aquatic animal health. An example of this was the classification of type two coldwater vibriosis which emerged as a significant problem for salmon production in Atlantic Canada in the mid-1990s. Research performed at our laboratory, in collaboration with a commercial vaccine producer, resulted in the identification and molecular classification of a novel *Vibrio* species (type 2 *Vibrio salmonicida*) which was able to infect fish previously vaccinated with the existing commercial *V. salmonicida* vaccine. As a result of this study the cold water vibriosis vaccine was reformulated to include the newly characterized bacterium and the disease was subsequently brought under control.[3]

Molecular methods have also found a substantial role in the development of novel vaccines based upon recombinant DNA technology. There are three broad generations/classes of recombinant vaccine. The first class of recombinant vaccines are sub-unit vaccines. Here, a sequence encoding a protein considered to rep-

resent a major protective antigen is cloned into a protein expression plasmid/vector which is then introduced into a surrogate expression host (most commonly *Escherichia coli*), to produce large quantities of recombinant protein, which following purification can be applied as a vaccine. At present, commercial sub-unit vaccines are limited to two products for the control of infectious pancreatic necrosis virus (IPNV). However, various other vaccines of this class have been reported in the scientific literature and it seems likely that more sub-unit products will emerge, mainly for viral diseases where a single antigen is often enough to stimulate high levels of protective immunity.

The second class of recombinant vaccines are live attenuated recombinant vaccines. An example of this class of vaccine is the live auxotrophic (*aro A*) mutant of *Aeromonas salmonicida* which was developed in 1993 for use as a furunculosis vaccine.[4] The same technology has subsequently been used to produce prototype vaccines against disease caused by *Aeromonas hydrophila* and *Edwardsiella ictaluri*. The mutation in the *aroA* phenotype was achieved through the replacement of the wild type gene with a defective copy of the gene using the technique of homologous recombination. The resulting mutant bacteria are unable to synthesize a number of aromatic amino acids and as such are unable to grow unless supplied with these metabolites, which are not present in fish tissue. When introduced into a fish, the bacterium initiates the infection process but only achieves limited replication as it quickly expends the limited quantities of the metabolites which it obtained when grown *in vitro*. Yet, this limited replication is sufficient to stimulate the fish immune system to generate a strong protective response towards *A. salmonicida*.[4] Despite showing great potential, this class of vaccine has not been received with much enthusiasm by the aquaculture industry. It seems likely that the main reason for this is an inherent lack of confidence in the inability of the mutant bacterium to revert to a pathogenic form. Such uncertainity is only one part of the myriad of concerns associated with the release of genetically modified organisms into aquatic environments where containment would be virtually impossible. The majority of these concerns are perceived rather than factual. Yet it is pertinent to consider that given our current, incomplete knowledge of how microbes interact in nature and the fact that genetic exchange/horizontal gene transfer between microbes does occur at high frequency, as exemplified by an ever increasing incidence of antibiotic resistance, the possibility (albeit remote), does exist that a live mutant strain could acquire the means through which to bypass the engineered deficiency and regain the ability to cause disease. For this motive, it seems very unlikely that live recombinant vaccines will find a role in the control of aquatic animal disease.

Nucleic acid vectors encoding pathogen antigens represent the third and most recently developed generation of recombinant vaccines to have entered the arena of aquatic animal health. The basic principle behind these nucleic acid vaccines (NAVs) involves the administration of non-infectious, recombinant nucleic acid carrying a gene encoding a protective antigen with expression under the control of a strong eukaryotic promoter.[5] It has been stated that this type of recombinant vaccine offers advantages over the other classes of recombinant vaccine. These advantages include relatively low production costs and the ability to easily produce multivalent vaccines by simple mixing of different vectors or through the design of vectors capable of expressing multiple antigens simultaneously. In addition, the stability of DNA removes the need for a cold chain when transporting or storing such vaccines. In general NAVs for use with aquatic animals are essentially the same, in terms of core vector structure, as those developed for use with terres-

trial animals and humans. The scientific literature contains many papers which testify as to the potency of NAVs for vaccination against a number of diseases of aquatic animals but to date no product has been licensed for commercial use. However, a large number of vaccines are under active development and it appears likely that these vaccines will prove invaluable in situations where conventional vaccines or sub-unit recombinant vaccines have failed to provide positive cost to benefit ratios. One such example is the salmonid disease infectious haematopoetic necrosis (IHN). Yet, it should be emphasized that a number of issues remain to be addressed before this technology will gain wide-scale acceptance. Among these is consumer resistance to the use of gene technology in the production of food for human consumption. In common with the release of live recombinant vaccines, the introduction of naked DNA into fish tissues carries with it a degree of perceived, rather than scientifically established, risks. Indeed, no scientific evidence of negative side effects have been documented.[6]

Conclusions

The use of molecular tools has become routine in many aspects of research into aquatic animal health. In the area of disease diagnosis, these methods—principally PCR—represent research tools rather than a means through which to achieve definitive diagnosis. The lack of field validation of most PCR assays must be addressed in order for this technique to become fully accepted by regulatory agencies as a tool for primary diagnosis. In contrast, the use of molecular typing methods for the establishment of databases has begun to emerge as a central component of disease management strategies and this trend seems set to continue. Recombinant DNA technology has allowed the development of new generations of vaccines which offer great potential for the control of diseases of aquatic animals. A number of obstacles, mainly in terms of consumer concerns over safety and genetic manipulation of food animals, will need to be addressed before these vaccines achieve wide-scale acceptance.

"Recombinant DNA technology has allowed the development of new generations of vaccines which offer great potential for the control of diseases of aquatic animals."

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Rick Rideout

Using Sperm Cryopreservation to Advance Marine Finfish Aquaculture

Rick M. Rideout, Edward A. Trippel and Matthew K. Litvak

Cryopreservation of sperm plays a major role in breeding programs implemented by the livestock industry. To a lesser extent, cryopreservation procedures have been developed to help with gamete management for heavily cultured fishes such as carps, tilapias and salmonids. With some exceptions (e.g., turbot), research on the cryopreservation of sperm from marine fishes has been less intense because these fishes have not been the focus of major culturing efforts. With the identification of numerous marine fishes as potential alternate species for aquaculture, however, has come the need to develop breeding programs and gamete management strategies. Here we describe sperm cryopreservation procedures developed for three potential aquaculture species (haddock *Melanogrammus aeglefinus*, Atlantic cod *Gadus morhua,* and winter flounder *Pseudopleuronectes americanus*) and explore how sperm cryopreservation can contribute to an effective breeding program.

Introduction

Cryopreservation involves the storage of living cells at very low temperatures, usually –196°C, the temperature of liquid nitrogen. At such temperatures, it is theorized that living cells can be stored indefinitely without deleterious effects. Determining the optimum procedure for freezing living cells can be very tedious. Using an inappropriate freezing rate will cause cryoinjuries to the cells (Fig. 1) and likely result in poor post-thaw survival. To help prevent these cryoinjuries, cells are pretreated with an isotonic medium, known as a diluent or extender, and a cryoprotecting agent, which helps prevent the formation of ice crystals within the cells. Cryoprotectant and diluent selection are only two of many factors that have to be considered to successfully cryopreserve living cells (Table 1). Determining the optimal freezing process is also complicated by the fact that interac-

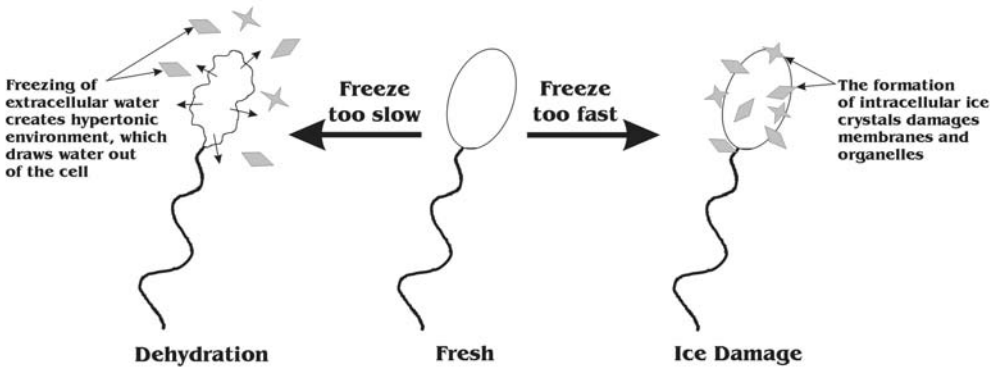

Figure 1
Typical cryoinjuries caused by not properly controlling freezing rate.

Table 1
Factors that must be considered when developing cryopreservation protocols for fish sperm.

Factor	Rationale
Timing of sperm collection	Sperm collected at different times of the year may be more or less amenable to cryopreservation.
Cryoprotectant selection	A cryoprotectant must provide protection against ice crystal formation but must also be non-toxic to the cells.
Cryoprotectant concentration	A higher concentration of cryoprotecting agent will provide greater protection against ice crystal formation but higher concentrations may be toxic.
Diluent selection	A diluent must provide an isotonic environment and must not activate sperm.
Dilution ratio	An appropriate ratio of sperm to diluent helps to control the rate of cellular dehydration during freezing.
Acclimation time	Various cryoprotectants, such as glycerol, are composed of large molecules and therefore may require longer sitting times prior to freezing to allow the chemical to penetrate the cells.
Freezing rate	Freezing cells too fast results in cell death due to damage caused by ice crystals. Freezing cells too slow results in cell death due to cellular dehydration.
Freezing vessel	The optimal freezing rate will depend, at least in part, on the surface area to volume ratio of the containers within which the sperm is frozen. Various sizes of cryogenic straws and vials are available commercially.
Thawing rate	Cells must be thawed quickly in order to prevent ice crystal formation.
Time between thaw and activation	The duration of viability of post-thaw sperm may be reduced in comparison to fresh sperm and therefore sperm should be used quickly after thawing.

tions may exist between the variables (e.g. a cryoprotectant that works well with one diluent may not work well with another). In many cases, cryopreservation protocols appear to be species specific.

The ability to cryopreserve sperm, eggs and embryos plays a vital role in the breeding programs of many species of shellfish, livestock and even humans.[1,2,7,13] However, attempts to cryopreserve fish eggs and embryos have been unsuccessful, perhaps because their large size, large amount of yolk and thick chorion make it difficult to achieve cryoprotectant penetration and a uniform freezing rate. Fish sperm, on the other hand, appear to be amenable to cryogenic storage. Procedures for cryopreserving fish sperm have been examined in the

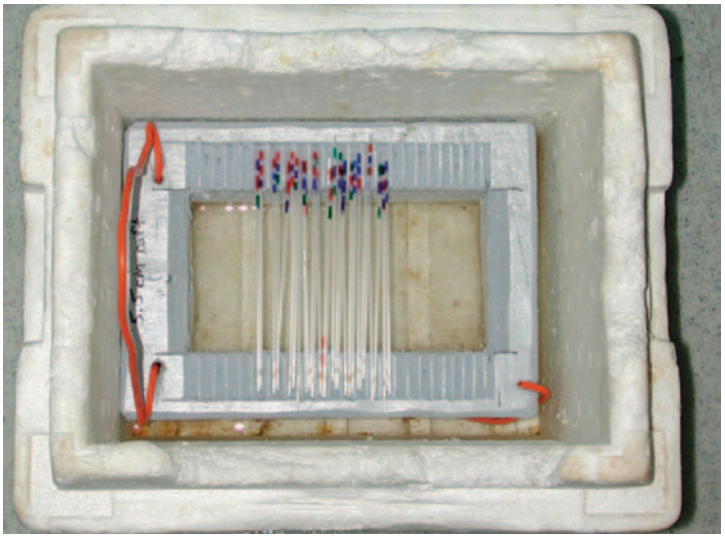

Figure 2
Samples are floated at a predetermined height above liquid nitrogen in order to control freezing rate. Note that straws are colour coded to indicate individual and/or cryogenic treatment.

greatest detail for the most heavily cultured species. In Canada, salmonids dominate the aquaculture industry and therefore considerable research has been directed at developing sperm cryopreservation protocols for species such as rainbow trout, Arctic charr and Atlantic salmon.[4,5,11] In recent years, attempts to diversify the Canadian aquaculture industry have led to the identification of numerous marine fish species that have potential for aquaculture development. Following the lead of the salmonid industry, researchers involved with these new aquaculture species are developing breeding programs that will help maximize production and prevent inbreeding depression. The cryopreservation of sperm has been identified as a technique that may help establish and maintain such breeding programs.

Cryopreserving Haddock, Atlantic Cod and Winter Flounder Spermatozoa

Based on experiments conducted at the St. Andrews Biological Station and the University of New Brunswick[9,10] a procedure has been developed for the cryopreservation of haddock (*Melanogrammus aeglfinus*), Atlantic cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) spermatozoa.

Sperm is collected using a syringe, making it easy to avoid urine-contaminated sperm and to eject accidentally collected contaminated sperm in order to prevent further contamination of the sample. Syringes filled with sperm are kept on crushed ice and cryogenically frozen within one hour of collection. In test tubes, sperm is diluted 1:3 with a sucrose-based diluent known as Mounib's[8] medium (0.125 M sucrose, 0.100 M $KHCO_3$, 0.0065M reduced glutathione). Propylene glycol is then added as cryoprotectant (10% of the total volume) and the sample is mixed by swirling.

In order to draw the sperm solution into 0.25 mL cryogenic straws (Minitube Canada), the straws are fitted one at a time into the opening of a small syringe, so that lifting the syringe plunger pulls the solution into the straw. Straws come with one end prefilled with cotton. Once the straw is filled with the sperm solution, the other end is sealed with a metal bead or clay. Straws are color coded to identify the male or, in the case of ex-

Figure 3
After reaching –90°C, sperm samples are stored in liquid nitrogen at -196°C.

perimental procedures, to indicate the treatment. The freezing rate is controlled by floating the straws 5.5 cm above liquid nitrogen on a styrofoam raft in a styrofoam box containing an inch or less of liquid nitrogen (Fig. 2). After 90 sec, straws are plunged directly into liquid nitrogen in a 50-L Dewar flask (Fig. 3). Because the canisters of the Dewar flask are too large and deep to allow easy removal of the small straws, sample containers to hold the straws were created using PVC pipe (Fig. 4). Several sample containers fit into each of the larger canisters.

To thaw sperm samples, a small number are removed at a time from the Dewar flask and placed immediately into a 30°C water bath for seven seconds. The straws are quickly dried and both ends are cut off using scissors, which allows the sperm solution to run out. Sperm samples are used to fertilize eggs as quickly as possible after thawing (usually within 10 to 15 min). Because the chemicals used to cryopreserve sperm may be toxic to the eggs, the sperm solution is thoroughly rinsed from the eggs once enough time has elapsed to allow fertilization to occur (approx. 1 min.).

Role of Sperm Cryopreservation in Finfish Aquaculture

The ability to indefinitely store fish sperm provides a degree of control in breeding programs that is not available using only natural spawning and short-term gamete storage techniques. The following is a list of potential applications of sperm cryopreservation to the development of aquaculture broodstock programs.

1. Banking of selectively bred traits

The goal of aquaculture (and indeed any culturing program) is to select for favorable phenotypic traits over a number of generations, including improved growth, food assimilation efficiency, and reduced aggression. The storage of gametes from these selectively-bred fish ensures the continued expression of these traits in future generations.

2. Maintaining control lines in selective breeding

To breed for selected traits, individuals that produce offspring with those favorable traits are selected as broodstock. Over multiple generations of selection based on favorable traits, however, the gene pool of the population may be reduced, which can lead to inbreeding depression. A simple method of reducing inbreeding depression in cultured species is to maintain a bank of sperm from the wild population from which the initial broodstock were selected and to periodically re-introduce some of this natural genetic variability into the culture population. This is analogous to periodically adding wild fish to the broodstock population, without the problems

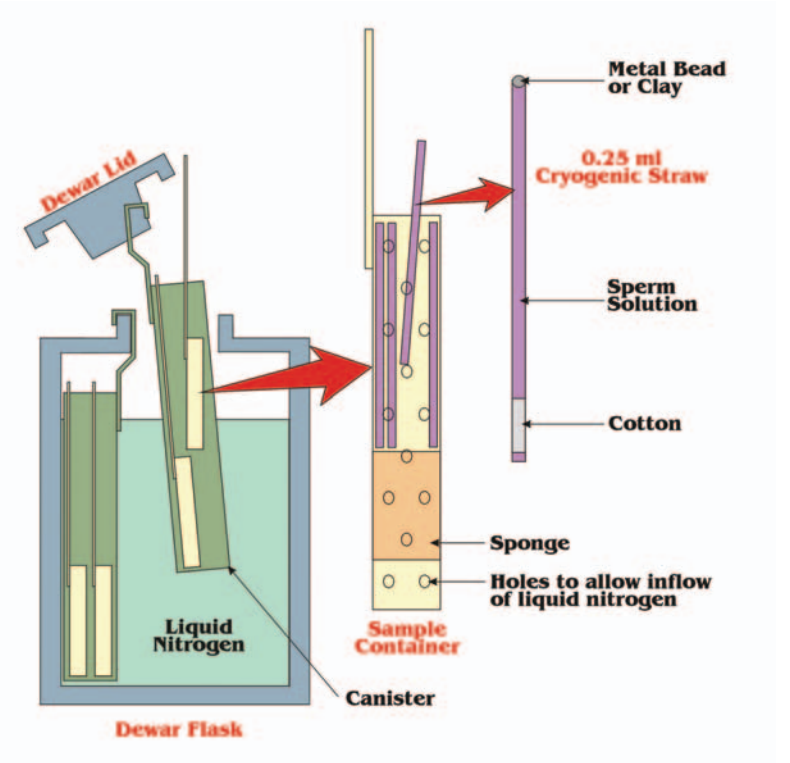

Figure 4
Illustration of the system used to cryogenically store fish spermatozoa at the St. Andrews Biological Station and the University of New Brunswick. Spermatozoa are frozen in straws, which are then inserted into sample containers and lowered into large canisters in a Dewar flask filled with liquid nitrogen.

of continually collecting wild fish and the potential for disease introduction.

3. Synchronizing male and female reproductive cycles

In attempts to maintain an active hatchery throughout the entire year, various techniques have been developed to alter the timing of spawning of fishes in captivity. These techniques include the alteration of photoperiod and/or temperature as well as hormonal treatments. The availability of cryopreserved sperm eliminates the need to alter spawning time of males and guarantees that sperm will be available whenever eggs are produced.

4. Synchronizing reproductive cycles of multiple species

In the same way that cryopreservation can synchronize the availability of gametes from both sexes, it also enables the simultaneous availability of gametes from two species that usually spawn at different times of the year and therefore may help in the production of hybrids. For aquaculture purposes, hybridization between two species is often desirable because the progeny may be sexually sterile and experience higher growth rates than individuals that become sexually mature.

5. Simplifying transport of gametes

Spermatozoa frozen in liquid nitrogen are easily transported between sites in small, portable Dewar flasks without worrying about transport time, oxygen depletion or physical damage during transport. This method of transport makes it easy to share gametes between hatcheries to reintroduce genetic diversity, and may reduce the need for the expensive and cumbersome practice of exchanging fish.

6. Avoiding seasonal reductions in sperm quality

In some marine fish, spawning occurs over an extended period of several months. Sperm collected late in the season may be of lower quality due to the effects of ageing.[12] To avoid the use of late season, low viability gametes, sperm could be collected and cryopreserved early in the season to have on hand when needed.

7. Banking of excess gametes

In fishes that produce only very minute amounts of spermatozoa, gamete conservation becomes very important to maximize production. Cryopreserving all available sperm ensures minimal wastage, and can be particularly economical and time-saving if the time has been taken to alter spermatozoa in one of the following manners:

- *Monosex gametes:* The administration of androgens to genetically female fish (i.e., XX) has been used to change them into functional males. The sperm produced by these fish lack a Y chromosome and therefore crossing these 'males' with normal females produces all-female progeny.
- *UV-treated gametes:* Sperm whose DNA has been rendered useless via ultraviolet light treatment can also be used to produce all female progeny.
- *Transgenic gametes:* Portions of DNA that code for favorable traits in other species can be spliced into the DNA of target species. If successful, the result of this transgenic is to give the target species traits that it previously did not have and which may make it more attractive to aquaculture. Cryopreserving the sperm of these transgenic fish is a means of storing the new genetic code.

The cryogenic storage of sperm that have received such biotechnological treatment would reduce the frequency with which such treatments have to be performed and could ultimately lead to economic savings.

8. Preventiing vertical disease transmission

It has been suggested that the cryogenic storage of gametes may prevent the vertical transmission of diseases (i.e., freezing kills pathogens) but this has yet to be confirmed.[6]

The above potential applications of sperm cryopreservation suggest that it could be a very powerful tool for aquaculture and the establishment of effective breeding programs. In fact, it is expected that the use of gamete cryopreservation as a means of storage will increase as the development of high-performance selectively bred or transgenic fishes increases.[3]

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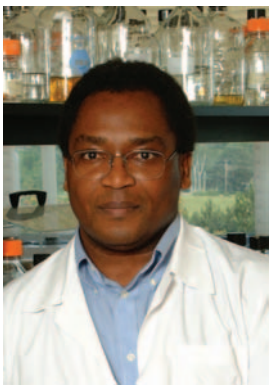


F. S. B. Kibenge

Molecular Pathology in ISAV Infection

Emeka E. Moneke, Tomy Joseph, Basil O. Ikede, and Frederick S. B. Kibenge

The current understanding on the pathogenesis and the mechanism of persistence of ISAV in fish is limited. Three permissive fish cell lines SHK-1, CHSE-214 and TO were used to determine if the cytopathic effect (CPE) observed in ISAV isolate NBISA01 and U5575-1 infection is due to apoptosis or necrosis. Apoptosis was observed only in SHK-1 and CHSE-214 infected cells with both isolates. TO cells infected by both isolates did not undergo apoptosis but showed damage characteristic of necrosis. These findings suggest that the mechanism of cell death during ISAV infection is dependent on the cell type. The TO cells were used to study the gene expression of ISAV using New Brunswick isolate-RPC-01-0593-01, Nova Scotia isolate-U5575-1 and Norwegian isolate 810/9/99 and in situ hybridization with ISAV segments 7 and 8 riboprobes. The difference in the frequency of hybridization signals between U5575-1 and the other two isolates was statistically significant (P = 0.004), indicating that the pathology associated with ISAV infection in vivo may depend not only on infected cell types but also on the infecting ISAV isolate.

Introduction

Infectious salmon anemia virus (ISAV) is the latest member of the family *Orthomyxoviridea*, genus *Isavirus*.[1,2] The virus causes infectious salmon anemia, a highly infectious clinical disease of marine farmed Atlantic salmon characterized by variable mortalities, exopthalmia, pale gills, ascites, hemorrhagic hepatocellular necrosis, and renal interstitial hemorrhage and nephrosis.[3, 4] The primary target cells of the virus in Atlantic salmon are endothelial cells, leucocytes, and macrophages.[5,6] The SHK-1,[7] TO[8] and ASK-2[9] cell lines developed from Atlantic salmon head kidney are used in the propagation of this virus. The three cell lines are macrophage-like; however, they differ in their growth characteristics and ISAV-induced CPE. Other cell lines such as CHSE-214,[10] AS[11] and Rtgill[12] are permissive to ISAV but visible CPE is observed only in CHSE-214 cells.[13]

ISAV isolates replicate differently in the permissive cell lines.[13,14] It was suggested that variation in the surface hemagglutinin protein,[15-17] could account for the difference in the virus phenotypes. Significant nucleotide and amino acid sequence differences in the hemagglutinin gene of isolates[17,18] lead to grouping into European and North American hemagglutinin genotypes. Differences were also reported in the pathology associated with different isolates of ISAV.[19, 20] Recently, we reported differences in ISH signals from different replicating ISAV isolates in infected fish tissues that appeared to suggest that Atlantic salmon infected with isolates of the two hemagglutinin genotypes show differences in the frequency and intensity of hybridization signals.[21]

Viruses cause cell death either by necrosis or apoptosis. Apoptosis, unlike ne-

crosis, is a programmed cell death that occurs as a result of receptor or non receptor-mediated signals[22] and characterized by cytoplasmic shrinkage, chromatin condensation and intranucleosomal cleavage into oligomers of 180- to 200-base pairs (bp) multiples, phosphatidylserine exposure, plasma membrane blebbing, cell fragmentation into apoptotic bodies which are phagocytosed by macro phages or other surrounding cells without provoking an inflammatory response.[23-25] Viruses can trigger and/or inhibit apoptosis in infected cells to their advantage by either blocking the apoptotic response to ensure efficient virus replication or induce apoptosis as an effective way for their dissemination by avoiding the host immune response.[24, 26] The present study characterizes the pattern of gene expression of ISAV segments 7 and 8 mRNA in fish cells infected with three ISAV isolates in relation to the mechanism of cell death.

Materials and Methods

Cells and viruses

The ISAV isolates Norway 810/9/99 and U5575-1 of European genotype, NBISA01 and RPC-01-0593-01 of North American genotype used for this study were propagated and titrated in TO cell line as described previously.[27]

Riboprobe preparation

Preparation of the ISAV segment 7 and 8 riboprobes were carried out as previously described.[6] Briefly, total RNA extracted from ISAV-infected cell culture lysate with Trizol Reagent (Invitrogen Life Technologies) was used in reverse transcription-polymerase chain reaction (RT-PCR) to obtain ISAV CDNA. The PCR primers consisted of ISAV RNA segment 7 (GenBank Acc. No. AX083264) forward primer 5 -ATG TCT GGA TTT AAC TCG AGG-3 (nucleotides 1-22) and reverse primer 5 -CAT AAC AAG TTT TCA ACC AAT C-3 (nucleotides 770-791), and ISAV RNA segment 8 (GenBank Acc. No. AF312317) forward primer 5 -GAAGA GTCAG GTGCC AAGACG-3 and reverse primer 5 -GAAGT CGATGA TCTG CAGCGA-3 . The RT-PCR products were first cloned in the pCR®II-TOPO® Vector (Invitrogen Life Technologies) then subcloned into pGEM-3Z vector (Promega) using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals). The in-vitro transcription reactions were carried out in presence of digoxigenin-11-deoxyuridine triphosphate as label. The specificity and sensitivity of transcripts were determined by Northern blot hybridization as previously described.[6]

Apoptosis assay

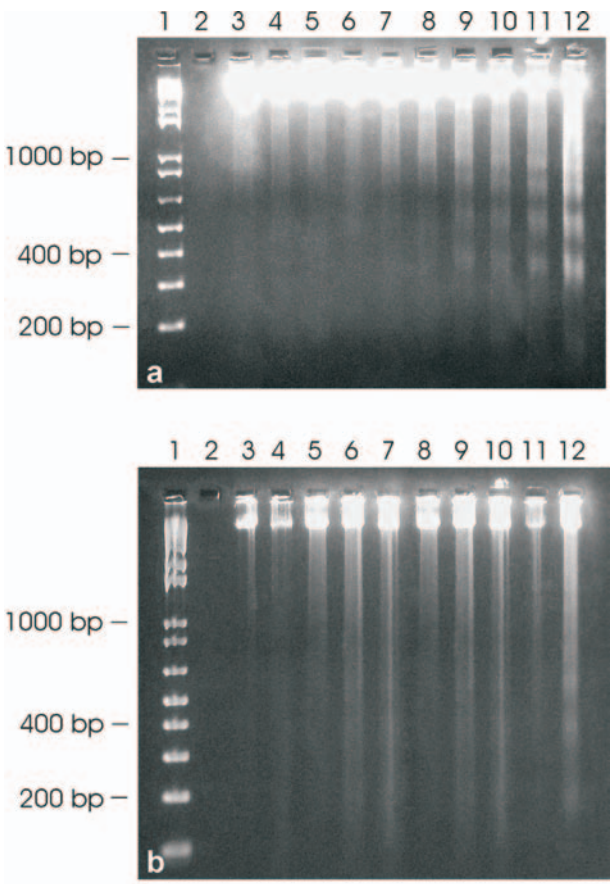
The CHSE-214, TO, and SHK-1 cells were each grown in a six-well tissue culture plate under appropriate conditions as previously described.[13,27] The cell monolayers were infected with an approximately 10 multiplicity of infection (moi) of ISAV isolate NBISA01 in maintenance medium. The infected cells were harvested as follows: for TO cells at 6, 12, and 18 hours and 1, 2, 3, 4, 5, 6, and 7 days post infection (dpi); for SHK-1 cells at 6, 12, and 18 hours and 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 dpi; for CHSE-214 cells at 1, 3, 5, 7, 9, 11, and 13 dpi. In a repeat experiment, SHK-1 and TO cells were infected with ISAV isolate U5575-1 and harvested at 12 hours, and 1, 2, 3, 4, 5, 6, and 7 dpi. The extent of study was based on development of CPE in each cell line as previously described.[13,27] Cellular DNA was isolated from harvested infected and uninfected cells using a previously described procedure.[28] The isolated cellular DNA was analyzed using a 1.8%

agarose gel electrophoresis for chromosomal DNA fragmentation. DNA fragmentation was confirmed by fragment end labeling assay using TdT-FragEL DNA fragmentation detection kit (Oncogene, San Diego, CA) according to manufacturer's instructions.

In situ hybridization (ISH) on infected TO cells

Confluent monolayers of TO cells were respectively infected with 0.2 ml of 1:100 dilution of ISAV isolates (U5575-1 and RPC-01-0593-01). The infected cells were harvested at 4, 8, 12 hours and at 1, 2, and 4 dpi. Uninfected controls cells were harvested at 4 hours, and at 1 and 4 hpi. Cells were checked for CPE before being harvested and fixed in 4% paraformaldehyde, and used for ISH. In a repeat experiment, three ISAV isolates (U5575-1, RPC-01-0593-01, and Norway 810/9/99) were used to infect TO cells at an moi of 10. The infected and control cells were harvested and fixed as before. ISH was performed on the fixed infected cells using the ISAV RNA segments 7 and 8 riboprobes following the procedure previously described.[6]

The frequency of ISH signals was analyzed by taking pictures of cells in 10 random fields at x16 objective. All cells with or without hybridization signals in five random fields were counted. The percent averages of cells with hybridization signals were calculated from the total cells in the five fields. A student *t* test and one-way analysis of variance (ANOVA) were used to test for the difference in the frequency of hybridization signals in cell cultures between different isolates and segments of ISAV.



Results

Riboprobe synthesis and specificity

The integrity, polarity and specificity of the riboprobes were checked as previously described.[6]

Apoptosis in ISAV-infected cells is cell-type specific

DNA fragmentation was observed in SHK-1 cells infected with ISAV strain NBISA01 beginning on 4 dpi, and with ISAV strain U5575-1 beginning on 6 dpi when CPE was apparent. The intensity of the DNA fragmentation increased (Fig.1a) as the CPE increased. No DNA fragmentation was observed in uninfected SHK-1 cells (Fig. 1b). DNA fragmentation was also observed in CHSE-214 cells infected with NBISA01 beginning on 9 dpi which corresponded with the appearance of CPE. No DNA fragmentation was observed in uninfected CHSE-214 cells. CHSE-214 cells infected with U5575-1 did not show either CPE or DNA fragmentation. No DNA fragmentation was observed in ISAV-infected and uninfected TO cells (data not

Figure 1

Agarose gel electrophoresis showing apoptosis in ISAV-infected cells. (a) SHK-1 cells infected with ISAV strain NBISA01 showing apoptotic DNA fragmentation. Lane 1: 1kb+ DNA ladder. Lane 2: empty. Lanes 3 to 12: DNA isolated from ISAV-infected SHK-1 cells at 6, 12, and 18 hours, and at 1,2,3,4,5,6 and 7 days post-infection.

Table 1. Comparison of hybridization signals using segment 7 riboprobe on TO cells infected with three ISAV isolates at different time intervals (nd denotes not done).

Groups	% average number (standard deviation) of positive cells in five fields at different times					
	4 hpi	8 hpi	12 hpi	1 dpi	2 dpi	4 dpi
uninfected	0	0	0	0	0	0
U5575-1 (NSC)	0	0	0	30.04 (5.73)	nd	54.44 (8.13)
Norway 810/9/99 (NOR)	0	0	0	27.93 (	nd	79.52 (8.01)
RPC/NB 01-0593-1 (RPC)	0	0	0	nd	69.23 (	87.01 (14.16)

shown) although the infected cells showed CPE by day 2 with NBISA01 and by day 4 with U5575-1 which progressed to completion by 5-7 dpi. Consistent with the above results, fragment end labeling assay for apoptosis also revealed apoptotic cells in the ISAV infected SHK-1 cells (data not shown). No apoptotic staining was detected in both uninfected and infected TO cells. Absence of apoptotic DNA fragmentation in ISAV-infected TO cells, which developed complete CPE, suggested that TO cells may be undergoing necrotic type of cell death.

The replication of ISAV isolates U5575-1, RPC-01-0593-01 and Norway 810/9/99 differ in infected TO cells. Distinct hybridization signals were observed with segments 7 and 8 riboprobes in the nucleus and cytoplasm of cells infected with each ISAV isolate from 24 hpi (Fig. 2a). Onset of CPE was detected from 2 dpi, lifting of cellular monolayer was observed in RPC-01-0593-01 and Norway 810/9/99 infected cells at 4 dpi, while the monolayer of U5575-1-infected cells was intact. On subjective comparison of hybridization signals between the two isolates at 1 and 2 dpi, signals appeared stronger in cells infected with RPC-01-0593-01 (Fig. 2b) than in cells infected with U5575-1 (Fig. 2a). By 4 dpi hybridization signals were present in all infected cells (Fig. 2c and 2d). In a repeat experiment ISH signals were observed in the TO cells as summarized in Tables 1 (segment 7) and 2 (segment 8).

Comparison of hybridization signals between the different isolates

At 1dpi, a significant difference was observed with Norway 810/9/99 infected cells showing more signals with segment 8 riboprobe than other isolates ($P = 0.001$). At 2 dpi, the signal frequency increased rapidly and significantly in RPC-01-0593-01 infected cells and by 4 dpi, no significant differences were observed with the two riboprobes in Norway 810/9/99 and RPC-01-0593-01 infected cells. By 4 dpi, the U5575-1 infected cells had a significantly lower frequency of signals with both riboprobes when compared with other two isolates ($P = 0.004$). Subjectively, the signal intensity in the U5575-1 infected cells appeared weaker compared to the other isolates. No difference was apparent in signal intensity between Norway 810/9/99 and RPC-01-0593-01 infected cells.

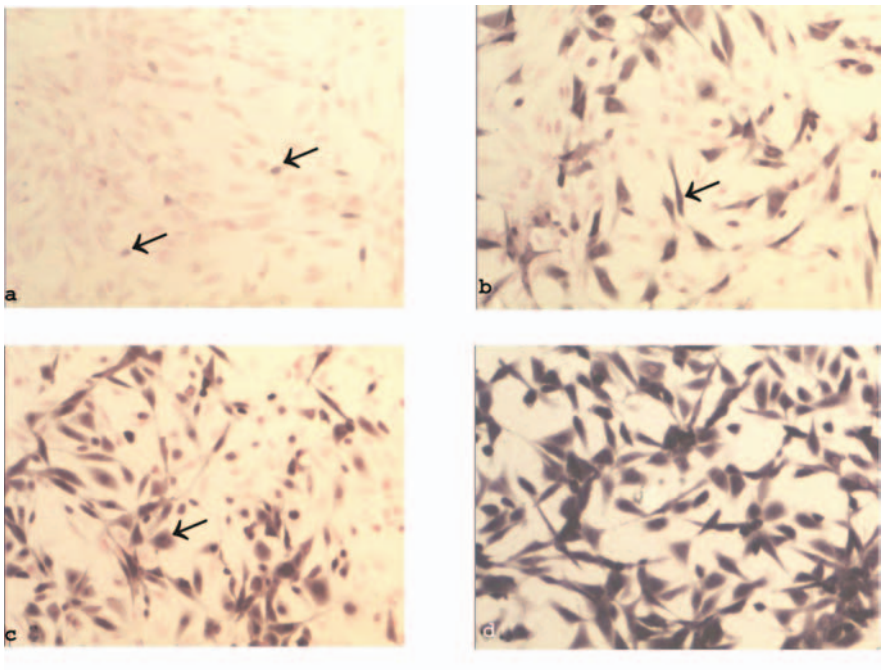
Comparison of signals between the riboprobes

At 1 dpi, significant difference was observed with segment 8 riboprobe showing more frequency in Norway 810/9/99 infected cells ($P = 0.011$). At 2 and 4 dpi, significantly more frequency of signals were observed with the segment 7 riboprobe in RPC-01-0593-01 infected cells than segment 8. No significant differences were observed at other times. Subjectively, the intensity of signals was greater with

Table 2. Comparison of hybridization signals using segment 8 riboprobe on TO cells infected with three ISAV isolates at different time intervals (nd denotes not done).

Groups	% average number (standard deviation) of positive cells in five fields at different times					
	4 hpi	8 hpi	12 hpi	1 dpi	2 dpi	4 dpi
uninfected	0	0	0	0	0	0
U5575-1 (NSC)	0	0	0	28.41 (11.06)	nd	53.03 (12.07)
Norway 810/9/99 (NOR)	0	0	0	48.65 (	nd	71.70 (15.50)
RPC/NB 01-0593-1 (RPC)	0	0	0	21.39 (	54.80 (	64.16 (6.39)

Figure 2
In situ hybridization using a riboprobe to segment 7 in TO cells infected with different isolates of ISAV (U5575-1 and RPC-01-0593-01). (a) ISAV U5575-1 infected cells 1 dpi showing signals in the nuclei (arrows). (b) RPC-01-0593- 01 infected cells 1 dpi showing signals in the nuclei and cytoplasm of more than half the cells in the monlayer (arrow). (c) U5575-1 infected cells 2 dpi showing signals (arrow) in more cells, similar to RPC-01-0593-01 infected cells at 1 dpi. (d) All cells infected with RPC-01-0593-01 isolate showed signals at 2 dpi.



segment 7 riboprobe than with segment 8 riboprobe at all sampling times.

Discussion

The mechanisms of cell death caused by ISAV infection have recently been studied.[29] The TO cell line consists of uniform leucocytes,[8] whereas the SHK-1 cell line contains at least two subpopulations of cells, one of which is fibroblast like.[9] The CHSE-214 cell line consists of fibroblast-like cells; only the ISAV-infected SHK-1 and CHSE-214 cells developed apoptosis. Although it is possible that the CPE observed in vitro may not be relevant to events in vivo, it is generally accepted that the execution of either apoptosis or necrosis in virus-infected cells reflects the pathogenicity of viruses.[24] We therefore speculate that ISAV infection leads to destruction of highly susceptible cells such as TO cells (leucocytes), possibly by necrosis. Infection of such cell types in vivo may lead to inflammatory reactions and subsequent immune response and this may explain the clinical disease and pathology during a natural infection. At the same time, ISAV is capable of inducing apoptosis in cells such as SHK-1 and CHSE-214 cells. Infection of such cell types in vivo may cause no inflammatory reactions, and therefore subclinical disease and virus persistence during a natural infection.

In order to better understand the rationale for the difference in hybridization signals (replication rates) between the virus isolates belonging to North American and European hemagglutinin genotyes,

the Norway 810/9/99 isolate that is genetically related to the U5575-1 isolate, but is more pathogenic,[30-32] was introduced into this study. The frequency and intensity of signals for Norway 810/9/99 and RPC-01-0593-01 isolates were similar. Also, CPE and cellular detachments occurred at the same time with both isolates, indicating they might be similar in pathogenicity for TO cells. On the other hand, the U5575-1 isolate showed slower progression of CPE, lower frequency and weaker intensity of signals than the RPC-01-0593-01 and Norway 810/9/99 isolates in TO cells coupled with the observation of DNA fragmentation 2 days later in SHK-1 cells compared to NBISA01, all suggest that it might be less pathogenic. These findings indicate that the differences in ISH signals (replication rate) and the CPE observed are dependent on the particular virus isolate and not hemagglutinin genotype. The greater signal intensity observed with segment 7 riboprobe suggests that its mRNA is expressed in higher amounts than that of segment 8 at any given time.

In conclusion, this study shows that the difference in the hybridization signals and replication of the different ISAV isolates in TO cells is not dependent on the hemagglutinin genotypes but on the biological properties of the individual viral isolate. The type of cell death induced by ISAV is cell-type specific, causing apoptosis in SHK-1 and CHSE-214 cells and necrosis in TO cells, which may have implications for the pathogenesis and persistence of ISAV in Atlantic salmon.

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Molecular Genetic Research on Cultured Oyster Species in Nova Scotia

Bénédikte Vercaemer

Bénédikte Vercaemer, Koren Spence, and Ellen Kenchington

The European oyster *(Ostrea edulis)* and the eastern/American oyster *(Crassostrea virginica)* have been part of the Nova Scotia aquaculture industry for decades. Recently, both species have suffered significant mortalities. European oyster hatcheries have experienced close to 100% larval mortality in each of the last 3 years. The reasons for this are not known, although water quality, disease, and inbreeding have been suggested as possible factors. American oyster populations in the Bras d'Or lakes have also undergone a decline in recent years, with illegal harvesting and degradation of habitat thought to be contributing factors. The problem was compounded by the discovery of MSX disease in the lakes in 2002.

Research projects are being conducted on both species through Fisheries and Oceans Canada, in collaboration with industry partners. Molecular genetic markers are being used to measure a variety of parameters, including genetic diversity, allelic richness, genetic distances, and population structure within both species. The goal is to use this information to help develop management and breeding programs to assist in the continued propagation of these species in Nova Scotia, as well as to investigate the utility of these genetic markers in the enforcement of transfer restrictions and area closures as conservation or disease control measures.

"European oyster hatcheries have experienced close to 100% larval mortality in each of the last 3 years. The reasons for this are not known . . . "

There are currently two projects underway at the Marine Aquatic Resources Biotechnology Laboratory which apply molecular genetic techniques to the study of the European oyster *(Ostrea edulis)* with ACRDP funding and to the study of the eastern oyster *(Crassostrea virginica)* with CBS Regulatory funding. This manuscript provides a brief overview of both projects.

Genetic Diversity of European Oyster Populations in Nova Scotia

Introduction

The European oyster was introduced to Nova Scotia for the aquaculture industry in the 1970s, using stocks imported from naturalized populations in Maine. After several successful years of hatchery spat production, the industry experienced 100% larval mortality in 2001, 2002, and 2003. One of the factors that may have contributed to the collapse is a suspected loss of genetic diversity due to the

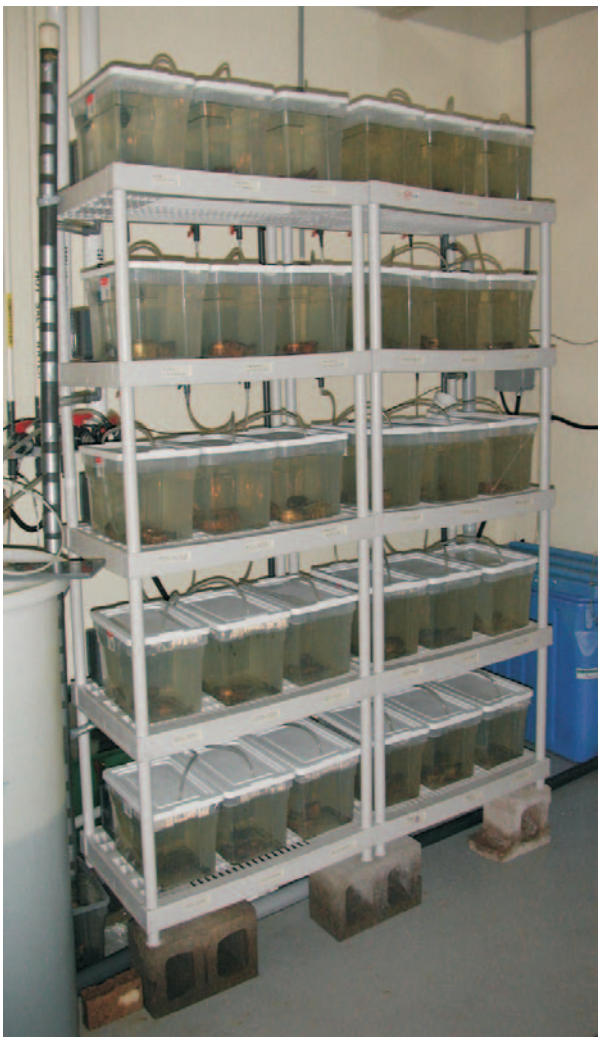
limited number of individuals used as foundation stock, possibly followed by inbreeding over subsequent years of hatchery propagation.

Research

In order to investigate the possibility that the Maritime hatchery stocks are suffering from a loss of genetic diversity, samples were taken from several hatcheries/grow-out sites around the province (Lunenburg, Cape Sable, and Port Medway), as well as from naturalized populations in the Maritimes (Sambro and Lake Lochard). Samples were also included from a cultured population in British Columbia (Okeover Inlet) and three naturalized populations from Maine (Blue Hill Bay, Cundy's Harbor, and Boothbay Harbor).

Over 700 individuals were genotyped using five microsatellite markers (OeduU2, OeduT5, OeduJ12, OeduH15 and OeduO9 loci) developed by IFREMER, France.[1] Data gathered were used to measure allelic frequencies, distribution and richness, observed versus expected heterozygosity, and genetic differentiation between sample locations.

Conditioning and spawning unit used for controlled mating of oysters in the hatchery at the Bedford Institute of Oceanography.



Results

Samples were amplified by PCR, using fluorescent-labelled primers. Products were run on ultra-thin, denaturing polyacrylamide gels, and visualized on a MJ BaseStation Fragment Analyzer.

Preliminary analysis showed a loss of allelic richness in the hatchery-propagated populations when compared to the naturalized populations in both Maine and the Maritimes. Although this indicates on-going genetic erosion in hatcheries, there remains a relatively high level of genetic diversity in these stocks.

The genetic differentiation between populations was examined using pairwise Fst values. These values were used to calculate co-ancestry genetic distances between populations.

These results support what is known of the history of the European oyster in this area. The Nova Scotia hatchery populations cluster together, reflecting their shared origin. The Lake Lochard naturalized population was established using oysters predominately from Lunenburg. The Sambro population was established using a hatchery stock that had been imported from Maine, via Dalhousie University, within the last few generations. This is reflected by their intermediate position between the Maine populations and the Nova Scotia hatchery and New Brunswick naturalized stocks.

Oysters from Maine were randomly selected for assignment testing using GeneClass. This program was able to accurately assign oysters to Maine populations rather than Maritime hatchery stocks based on the microsatellite profiles. This has the potential utility of

Comparisons Between Populations		Number of Individuals Amplified	Repeat Pattern	Observed Allele Size Range	Number of Alleles per Locus
Ostrea edulis					
NS hatcheries/Maritimes/Maine		232/241/254			
Locus	*Oedu*U2		(AC) (AG)	158-214	18/25/28
	*Oedu*T5		(CA)	106-174	17/19/27
	*Oedu*H15		(ATCT)	175-227	9/12/14
Crassostrea virginica					
Bras d'Or/Gulf of St. Lawrence		1194/219			
Locus	*Cvi*6		(GTTT)	165-247	13/16
	*Cvi*12		(CAAA)	90-150	10/18
	*Cvi*2i24		(CAAT)	369-501	10/12

detecting illegal importation of oysters from Maine, a *Bonamia*-infected region, to Nova Scotia which is currently *Bonamia*-free.

Molecular Tools for the Enforcement of Fisheries and Aquaculture Regulations and Assessment of Biodiversity for the American Oyster in Cape Breton, Nova Scotia

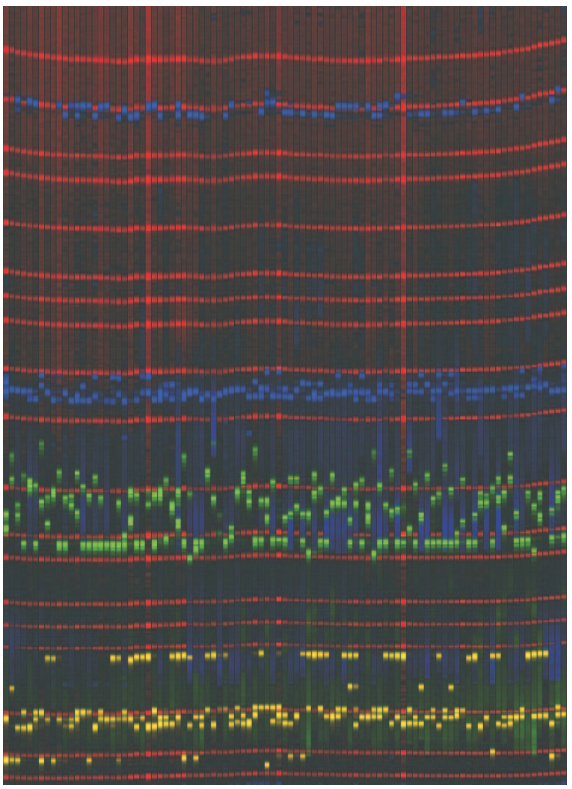
Introduction

The American Oyster is an economically, ecologically, and culturally important species in Cape Breton, but populations have been in decline in recent years, primarily due to over-fishing, illegal harvesting, and degradation of habitat. The appearance of MSX disease in the Bras d'Or Lakes in 2002 has also contributed to concerns about the future of the American oyster in this area, in both natural and aquaculture populations. Rejuvenation of depleted private leases and public beds through seeding and cultivation programs has been proposed as a solution by both DFO and the Eskasoni Fish and Wildlife Commission. However, maintenance of genetic diversity is essential in providing these populations with the resilience to adapt to environmental changes, including the appearance of MSX.

Research

Starting in 2003, samples have been taken annually from 6 to 10 sites. These sites include private leases as well as natural populations throughout the lakes. Samples are being screened at multiple microsatellite loci, and genotypes are used to create a database of genetic profiles which can be used to assess genetic diversity, to map pop-

Gel picture of American oyster samples run at 4 microsatellite loci, labelled in three dyes (HEX, FAM, and TET). The loci are *Cvi*12[(2)] and Cvi1g3, 2g14, and 2i23.[(3)]



ulation structure in the lakes, and to make informed management decisions. The database could also be used to aid in the enforcement of bans on transfers between MSX-infected and MSX-naive areas, as well as the enforcement of illegal harvesting of oysters from areas closed for conservation or public safety concerns.

Results

Currently, 12 microsatellite loci published by Brown[2] and Reece[3] have been selected and PCR and electrophoresis conditions have been optimized for the platforms available at MARBL. To control costs of genotyping, trials were performed to maximize the number of loci that can be run per gel. As a result, all 12 loci can be run on three denaturing polyacrylamide gels, greatly reducing the time and expense of genotyping.

Over 800 oysters have been genotyped at these loci. The second year of sampling at these locations is scheduled to begin in June, 2004.

Future Directions

- Continue to use molecular techniques to address issues of genetics, disease, and sustainable production in European and American oyster culture
- Create family lines through controlled crosses of European oysters within and between naturalized populations and aquaculture stocks in Nova Scotia
- Continue work on development of AFLP and SNP techniques for use with oyster species
- Create a multi-use database including genotypes, biological and morphological data, and ecological information relating to the American oyster in Cape Breton, NS.

Note

The results from the first year of the European oyster project were published in the Canadian Technical Report of Fisheries and Aquatic Science No. 24534 in 2003. A final report will be submitted in the fall of 2004. The American oyster project will continue for the next two years, culminating in a final report in 2006.

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