ANNUAL REPORT

NORTHEASTERN REGIONAL AQUACULTURE CENTER

For the Period
September 1, 2004 to August 31, 2006

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In cooperation with USDA/CSREES

Northeastern Regional Aquaculture Center Annual Report
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Introduction

The northeastern Regional Aquaculture Center is one of five Regional Aquaculture Centers established by the U.S. Congress under title XIV of the Agriculture and Food Act of 1980 and the Food Security Act of 1985. These centers, located in the northeast, southern, north central, western, and the topical/sub-tropical Pacific regions are administered by the U.S. Department of Agriculture, Cooperative States Research, Education and Extension Service (CSREES). Located at universities and/or research institution the regional center's mission is the support aquaculture research, development, demonstration, and extension education to enhance viable and profitable U.S. aquaculture production, processing and distribution which will benefit consumers, producers, service industries, and the American economy.

Organization and Administration

Regional Centers

The Regional Aquaculture Centers are administrative rather than physical centers. The Centers provide a means of assessing research and extension needs, assuring industry input, establishing priorities, and implementing aquaculture research and extension programs. The Centers facilitate implementation, administration, and coordination of regional research and extension programs, and they foster information exchange, research and extension linkages, and cross fertilization of ideas within and between regions and between organizations.

Organization

The Northeastern Regional Aquaculture Center (NRAC) has an administrative staff consisting of a one-half time Director, an Administrative Assistant, and a Coordinator. NRAC's Board of Directors (BOD) is the policy making body for NRAC and consists of nine members representing the Regional Agriculture Experiment Directors, the Regional Extension Directors, the 1890 Schools, Aquaculture Laboratories of the USDA, Agricultural Research Service laboratories in the Northeast Region, Sea Grant Directors, and industry. The BOD also has responsibility for approval of all NRAC projects. The BOD is assisted by an Industry (IAC) and a Technical (TAC) Advisory Committee. The IAC with assistance from the TAC summarizes industry research and extension priorities for the Northeastern Regional Aquaculture Industry and assists in assuring these priorities are incorporated into NRAC planning. The TAC with help from the IAC assists NRAC in assuring high quality projects having good science and addressing industry priorities are funded by NRAC.

The IAC and TAC are both comprised of one representative from each of the 12 states in the Northeastern Region and the District of Columbia. These states include Connecticut, Delaware, the District of Columbia, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, and West Virginia. Thus, there are 13 members on each committee who provide representation from all parts of the region and for the various sectors of the aquaculture industry. The TAC is divided into representatives from the research and the extension communities who provide their expertise to NRAC in defining priorities and selecting high quality research and extension projects.

Administrative Operations

The Northeastern Regional Aquaculture Center was located at the University of Massachusetts Dartmouth from 1988 until 2005. At that time the University of Massachusetts Dartmouth decided their priorities had changed and no longer wished to host NRAC. Through a competitive process the University of Maryland was selected by USDA, CSREES (Cooperative States Research, Education and Extension Service) to host NRAC and in December of 2005 NRAC was transferred to the University of Maryland at College Park, Maryland. The University of Massachusetts Dartmouth and the University of Maryland have been working to complete transfer of NRAC to the University of Maryland through 2005 and 2006. Because of constraints on funding FY 2006 funds were the first funds coming directly from USDA to the University of Maryland. When projects are completed and NRAC funds held at the University of Massachusetts Dartmouth are expended, the University of Maryland will become responsible for all of NRAC activities and the University of Massachusetts will phase out of NRAC activities.

All NRAC staff members are at the University of Maryland and the day to day operations of NRAC are operating out of the University of Maryland. The NRAC Director reports to the Dean of the College of Agriculture and Natural Resources, University of Maryland at College Park.

Board of Directors

The BOD members serve four year terms except for some of the initial BOD members who will have shorter terms to develop the staggered terms needed to provide continuity over time. The current BOD members are listed in Table 1.

Table 1. Members of the NRAC Board of Directors

Board Member	Representing	State Where Located
Dr. Reginal Harrell (Board Chair)	Dean, University of Maryland	Maryland
Dr. Dyremple Marsh	1890 Land Grants	Delaware
Dr. William Wolters	ARS	Maine
Dr. Barry Costa-Pierce	Sea Grant	Rhode Island
Dr. David Bengston	Experiment Stations	Rhode Island
Dr. Michael Timmons	Experiment Stations	New York
Dr. Richard Rhodes	Extension Directors	Rhode Island
Dr. Dennis Calvin	Extension Directors	Pennsylvania
Mr. Sabastian Belle	Industry	Maine

Industry Advisory Committee

The IAC is comprised of representatives from the District of Columbia and the 12 states in the Northeast Region. They serve three-year terms except for the first IAC which will have varying length appointments to develop the staggered terms needed to provide continuity for the IAC. Current members of IAC are given in Table 2.

Table 2. Members of the NRAC Industry Advisory Committee

IAC Member	Organization	State
Dr. Edwin Rhodes	East Coast shellfish Growers Association	Connecticut
Mr. Phil Shamback	Southern States	Delaware
Mr. Michael Pietrak	Maine Aquaculture Association	Maine
Mr. Richard Shuck	Maryland Aquatic Nurseries, Inc.	Maryland
Mr. Steven Wright	Chatham Shellfish Company	Massachusetts
Vacant		New Hampshire
Mr. George W. Mathis (Co-Chair)	Atlantic Coast Shellfisheries Council	New Jersey
Vacant		New York
Mr. Charles A. Conklin	Big Brown Fish Hatchery	Pennsylvania
Mr. Perry Raso	Ocean State Aquaculture	Rhode Island
Vacant		Vermont
Mr. William Hare	Cooperative Extension Service	Washington, D. C.
Mr. Mike Nardella	Rainbow Head Farms	West Virginia

Technical Advisory Committee

The TAC is comprised of representatives from the District of Columbia and the 12 states in the Northeast Region. They serve three year terms except for the first TAC which will have varying appointments to develop the staggered terms needed to provide continuity for the TAC. The TAC is divided into two groups with approximately one-half of the members representing research and approximately one-half representing extension. Current members of TAC are given in Table 3.

Table 3. Members of the NRAC Technical Advisory Committee

TAC Member	State	Extension/ Research
Dr. Sylvain Deguise	Connecticut	Research
Dr. Dennis McIntosh	Delaware	Extension
Dr. ChristopherV. Davis (Chair)	Maine	Research
Dr. Andrew Lazur	Maryland	Extension
Mr. Scott Lindell	Massachusetts	Research
Mr. Rollie Barnaby	New Hampshire	Extension
Mr. George E. Flimlin, Jr.	New Jersey	Extension
Ms. Laurie Trotta	New York	Research
Ms. Ann M. Faulds	Pennsylvania	Research
Dr. Dale F. Leavitt (Co-Chair)	Rhode Island	Extension
Dr. Maxwell Fairweather	District of Columbia (UDC)	Research
Dr. Kenneth Semmens	West Virginia	Extension
Vacant	Vermont	Research

Project Development

NRAC has two methods to develop projects: 1) the RFA method and 2) the project team method. The IAC develops priorities and the TIAC (IAC and TAC together) develop problem statements to convert the priorities into researchable statements. The problem statements are distributed throughout the Northeast Region with a RFA (Request for Applications). Thus, anyone interested in submitting a proposal may submit a proposal as long as it addresses the problem statements. In some situations there will be a pre-proposal stage and then only selected (by the TIAC) pre-proposals will be invited to submit full proposals.

The RFA method is the most common method used by NRAC. The project team is a process where a priority is defined, a problem statement is prepared and a request for a statement of interest is distributed throughout the Northeast Region. People responding to the statement of interest are then brought together to develop a proposal to address the problem statement. The project team method tends to work well in some situations such for extension projects. Currently the Northeast Regional Extension Project is the only project for which NRAC has used the project team method.

Current Activities

Administration of NRAC

The transition of the NRAC from the University of Massachusetts Dartmouth to the University of Maryland has been completed except for some of the funding that still remains at the University of Massachusetts. It is anticipated that the funds at the University of Massachusetts will be completely committed in 2007 and will be expended by approximately 2009. The University of Maryland has provided remodeled office space for NRAC. The one-half time NRAC Director and a full time Coordinator and Administrative Assistant have been hired at the University of Maryland. All of the contracting and administration of NRAC funds are now handled from the University of Maryland. Funding prior to FY 2006 was provided from USDA, CSREES to the University of Massachusetts Dartmouth. From FY 2006 and forward all funding will come from USDA, CSREES directly to the Center at the University of Maryland.

Projects 2005-2006

NRAC's year runs from September 1 to August 30 of each year. This report covers two years from September 2004 through August 2006. During this period NRAC has provided funding to 20 research and extension projects in addition to administrative projects and will be funding another set of projects that will start in 2007. In the last 18 months NRAC has committed over $2 million to projects and NRAC operations. Completion or project progress reports are included in this document for projects that have been in existence long enough to have submitted a progress or final report. Because of the NRAC transition from the University of Massachusetts Dartmouth to the University of Maryland has occurred during the period of this report some project reports were submitted to the University of Massachusetts Dartmouth and some to the University of Maryland. Most of the reports included in this publication have been submitted to the University of Maryland.

Table 4 lists the projects by title and total project funding level. Details of the projects including project titles, abstracts, total funding, project numbers, and project results and findings to date are available in the appendix of this report. Publications, videos, extension publications, and other written or visual materials produced as part of each project are listed to the extent available for each project. Although attempts were made to be as complete as possible some publications that resulted from NRAC funding, particularly papers presented and papers published in peer reviewed literature, may not be included due to the time lag between the end of a project and the publication of results.

Accomplishments

Transition to University of Maryland

Considerable effort has been required to make the transition of NRAC from the University of Massachusetts Dartmouth to the University of Maryland. A new office area has been completed and the NRAC staff has move into the space at Maryland. The organization of NRAC has been changed with the appointment of a new and smaller Board of Directors, a new TAC and new IAC. The BOD has been reduced in size from approximately 28 members to 9 members while the TAC and IAC are composed of 13 members each. New operating procedures for the BOD, TAC and IAC have been developed and for the most part implemented. A new procedures manual is approaching completion and will be distributed as soon as the BOD reviews and approves the manual.

New staff has been hired at the University of Maryland and the staff at the University of Massachusetts Dartmouth has moved or been transferred to other positions. Ms. Julie Smith is the only long time NRAC employee still involved in the accounting and contracting at the University of Massachusetts. The University of Massachusetts Dartmouth has a new Director of their Office of Research Grants, Ms. Joanne Zanella-Litke, who assists as necessary on subcontracts to the University of Maryland. All of the people involved at the University of Maryland are experienced employees but are new to NRAC. They include the Director, Dr. Fred Wheaton; the Coordinator, Ms. Sharon Adams; and the Administrative Assistant, Ms. Lou Jean Jackson. Mr. Christopher Aubry at the University of Maryland Office of Research Administration and Advancement has also been instrumental in the transition of NRAC from Massachusetts to Maryland.

Extension Program

The Northeast Regional Extension team has refocused their efforts and has met and developed a proposal for one year that has been funded. During development of the proposal the extension team, consisting of approximately 20 people from the Northeast Region have met four times and developed a strong plan for an active extension effort in the Northeast. Their initial efforts have been to become acquainted with each other and each person's state programs. They have developed four teams to concentrate on four separate areas. These teams are: publications, needs assessment, web site development and education, and outreach/conferences. Each team is developing deliverables and a plan for future development of aquaculture extension in their area of responsibility for the northeast for the future.

Table 4. NRAC Projects Active During the 2004-2006 Fiscal Years

Project Title	Total Budget	Start Date	End Date
Evaluation of Hard Clam, *Mercenaria mercenaria*, Stocks for QPX-resistance	$71,173.00	6/15/2006	6/15/2008
Cross Breeding and Field Trials of Disease-Resistant Oysters	$248,436.00	6/15/2006	6/15/2008
Economic Analysis of an Alternative Raceway Material--WVU	$73,996.00	7/1/2006	6/30/2007
Nutrition Studies in Hatchery Technology of Clownfish and Pygmy Angelfish Culture in Closed Marine Systems	$167,381.00	12/15/2004	12/29/2007
Development of Broodstock Diets for Atlantic Halibut	$32,054.00	7/1/2006	6/30/2007
National Aquaculture Extension Conference	$20,000.00	11/1/2006	8/15/2007
Development of Environmental Code of Practice and BMPs for East Coast Shellfish Growers	$220,114.00	1/1/2007	12/31/2008
Evaluating Restoration and Mitigation of Aquatic Plant Species and Markets to Advance Commercialization of the Industry	$449,903.00	1/1/2007	about 12/31/2009
NRAC Regional Extension Project	$102,682.00	11/1/2006	10/31/2007
Development of Diets & Rearing Conditions for Commercial Aquaculture of Black Sea Bass	$158,840.00	5/1/2002	4/30/2005
HFRP Floating Raceway to Raise Trout in Treated Mine Water	$48,000.00	11/1/2002	10/31/2005
Development of Culture Methods of Commercial Production of Rainbow Smelt *(Osmerus mordax)* (Berlinsky)	$126,208.00	1/1/2003	4/5/2006
Production & Culture Performance of Triploid Bay Scallops	$103,022.00	1/1/2003	7/31/2005
Increasing Hatchery Efficiency for Summer Flounder and Cod	$57,936.00	12/21/2003	12/20/2005
Development of Diagnostic and Management Techniques to Select Cod Brookstocks and Hatchery Stocks Free from Nodavirus	$124,612.00	11/1/2003	10/31/2005
Mini-Regional Extension Project to Develop and Deliver Industry Targeted Workshops--Phase II & Phase III	$30,850.00	9/1/2004	8/15/2005
Developing and Testing Novel Methodology for Land and Near Shore-based Aquaculture of the Green Sea Urchin	$169,650.00	1/1/2004	12/31/2006
Development of genetic Markers to Assess Disease Resistance in the Eastern Oyster	$128,486.00	2/1/2005	1/31/2007
Effect of Temperature on the Infection of Hard Clams (*Mercenaria mercenaria*) by the Protistan Organism, QPX	$154,805.00	9/1/2006	3/14/2008
Salmon Hatchery Effluent Management Utilizing Integrated Polyculture Technologies (Levine,	$15,000.00	1/1/2004	8/1/2006

Conferences

NRAC was a co-sponsor at the Northeast Aquaculture Conference and Exposition (NACE), one of the most important aquaculture conferences in the Northeast region. NRAC also co-sponsored the National Aquaculture Conference in Cincinnati, Ohio, a meeting that brings all of the aquaculture extension people from all over the U.S. together to network, share expertise, learn from extension methods presentations, share experiences, and learn what does and does not work in extension methods.

The NRAC Director provided presentations on NRAC activities at the NACE meeting, the National Trout Association meeting, and the Pennsylvania Aquaculture Association meeting. Mr. John Ewart presented a historical perspective on aquaculture extension in the Northeast region at the National Aquaculture Extension Conference.

Research Impact

The ability to produce triploid bay scallops was demonstrated and the triploid scallops were shown to provide superior yield compared to diploid scallops.

Showed that *Prophyra yezoensis,* a marine algae that has one of the highest growth rates of all plants, can be grown in very low salinity water (0.125 ppt) supplemented with divalent cations. Thus, this algae, which produces several highly salable pigments and long-chain polyunsaturated fatty acids, may have use as a nutrient uptake organism from aquaculture systems in a wide range of salinities.

Developed an effective methodology to cryopreserve rainbow smelt (*Osmerus mordax*) sperm that produces viability upon thawing similar to that of fresh sperm.

Developed methods to spawn wild-caught smelt (*Osmerus mordex*) with high fertilization rates (>95%) and high hatch rates (>80 %). Grow out methods were developed up to 60 - 110 mm in length but the results were more variable. These results will allow culture of rainbow smelt by growers for the baitfish market.

Developed a real time RT-PCR assay for nodavirus in cod and used this assay to determine if cod could be shipped from hatcheries. Negative results from the assay prevented several substantial lots of fish from having to be destroyed.

Cod egg disinfection technology for nodavirus based on iodine and/or ozone was developed to disinfect cod eggs.

Publications

Green Sea Urchin

Böttger, S.A., C.W. Walker CW., Unuma, T 2004: Care and Maintenance of adult echinoderms. Chapter 2. In development of sea urchins, ascidians, and other invertebrate deuterostomes: experimental approaches. Eds C.A. Ettensohn, G.M. Wessel, and G.A. Wray , Methods in Cell Biology 74: 17-38.

Walker, C.W., L. H. Harrington, M.P. Lesser, W.R. Fagerberg, 2005 Nutritive phagocyte incubation chambers provide a structural and nutritive microenvironment for germ cells of *Strongylocentrotus droebachiensis*, the green sea urchin. Biol Bull 209: 31-48.

Walker C.W., T. Unuma, M.P. Lesser, 2006. Gametogenesis and reproduction of sea urchins. In: J. Lawrence, (Ed.), 2nd edition, Edible Sea Urchins: Biology and Ecology. Elsevier, Amsterdam, The Netherlands, Develop Aqua and Fish Sci, 37: 11-33.

Böttger, M S, A..G. Devin, C.W. Walker. 2006. Suspension of annual gametogenesis in North American green sea urchins (*Strongylocentrotus droebachiensis*) experiencing invariant photoperiod - Applications for land-based aquaculture. Aquaculture 261:1422-1431.

Harrington, L.H., C.W. Walker, M.P. Lesser, 2007. Gametogenesis in the green sea urchin, *Strongylocentrotus droebachiensis*. Invert. Biol. (In Press).

Böttger, S.A. , C. E. Eno, C. W. Walker. Novel methodology for generating triploid green sea urchins – applications for open-ocean aquaculture. (in preparation for Aquaculture).

Böttger, S.A., M. L. Powell, S.A. Watts, Lawrence, AL, C.W.Walker. Application of photoperiod manipulation and new extruded diets in aquaculture of the green sea urchin (*Strongylocentrotus droebachiensis*). (in preparation for Aquaculture).

Barry, T. M., K. L. Carlton, B.R. Paolella, M.P. Lesser, S.A. Böttger, C.W. Walker. First echinoderm opsin identified from sea urchin tubefeet. (in preparation for Gene).

Bay Scallops

Zarnoch C., S. Hoffman, M. Schreibman, A. Surier, and R. Karney. 2005. The biochemical composition and adductor muscle cell size in diploid and triploid bay scallops, *Argopecten irradians*. Poster presented at the Brooklyn College Science Day; 17 May 2005.

Surier, A. R., R. Karney, C. Zarnoch, K. Tetrault, R. Rheault, X. Guo, Y. Wang, B. Walton. 2006. Impact of Triploidy on growth, shape, muscle size, biochemical composition and fiber size in the bay scallop *Argopecten irradians*. Poster for the National Shellfisheries Association conference 06, Monterey, California 2006.

Manuscripts:
Barton, B. 2003. Comparison of growth and survival rates of diploid and triploid bay scallop (*Argopecten irradians*) larvae induced with 6-dimethylaminopurine chemical treatment. M.S. thesis. North Atlantic Fisheries College, Port Arthur, U.K.

Maza-Iglesias M. 2004. Triploid *Argopecten irradians*: methods, biological consequences and value for the shellfish industry. M.S. Thesis. School of Ocean Sciences, University of Bangor, Wales, UK.

Papers Presented:
Surier, A. and Richard C. Karney, 2004. Induced triploidy in the Bay Scallops *Argopecten irradians* and early field culture performance presentation for World Aquaculture'04, Honolulu, Hawaii, USA.

Surier, A. and Richard C. Karney, 2004. Induced triploidy in the Bay Scallops *Argopecten irradians* and early field culture performance Presentation for the Milford Aquaculture Conference, New Haven, Connecticut.

Surier, A., Richard C. Karney, Kim Tetrault and Robert Rheault. 2004. Induced triploidy in the bay scallop *Argopecten irradians,* and field culture performance. Poster for Northeast Aquaculture Conference and Exposition, Manchester, New Hampshire.

Surier A., Richard C. Karney, Kim Tetrault and Robert Rheault. 2005. Induced triploidy in the bay scallop *Argopecten irradians,* and field culture performance. Presentation for Aquaculture America 05, New Orleans, Louisiana.

Surier, A., Richard C. Karney, Kim Tetrault and Robert Rheault. 2005. Induced triploidy in the Bay Scallop *Argopecten irradians* and field culture performance. Presentation for the Milford Aquaculture Conference, New Haven, Connecticut.

Surier,A., Rick Karney, Chester Zarnoch, Kim Tetrault, Robert Rheault, Dr. Ximing Guo , Dr. Yongping Wang and Bethany Walton. 2006**.** Impact of triploidy on growth, shape, muscle size, biochemical composition and fiber size in the bay scallop, *Argopecten irradiant.* Poster for National Shellfisheries Association Conference, Monterey, CA.

Nodavirus

Roberts, Steven and Rick Goetz. 2004. Quantitative real-time molecular methods for disease detection in marine fish. Presentation at Aquaculture '04, Honolulu, Hawaii March 1-5.

Roberts, Steven and Scott Lindell. 2004. Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus. Northeast Aquaculture Cnference and Exposition. Manchester, New Hampshire.

Lindell, Scott and Steven Roberts. 2004. Egg Disinfection and diagnostic techniques for Atlantic cod nodavirus. Nodavirus II Workshop, Portsmouth, New Hampshire, December 8.

Roberts, Steven. 2005. Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus. NRAC Annual Aquaculture Research, Extension and Business Opportunities Update, Dartmouth, Massachusetts, February 24.

Roberts, Steven. 2005. New molecular techniques to detect nodaviruses in Atlantic cod hatcheries, Global Aquaculture Advocate, April. 2pp.

Rainbow Smelt

Ayer, M.H., C. Benton, W. King, V. J. Kneebone, S. Elzey, M. Toran, K. Grange and D.L. Berlinsky. 2005. Development of practical culture methods for rainbow smelt *Osmerus mordax* larvae. North American Journal of Aquaculture 67:202-209.

DeGraaf , J.D. and D.L. Berlinsky. 2004. Cryogenic and refrigerated storage of rainbow smelt (*Osmerus mordax)* spermatozoa. Journal of the World Aquaculture Society 35(2):244-231.

Porphyra

Levine I. 2006. Case study on *Porphyra* cultivation in Maine, USA and New Brunswick, Canada. In: Aquaculture Compendium, Online at www.cabicompendium.org/ac. Wallingford, UK: CAB International. pp 1-13.

Presentations

Levine, I., D., D. Cheney 2005. *Porphyra* from sea to freshwater; osmoregulation in marine algae. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 19.

Liu, Y. C. , T Hogan, A. Cary, A. Silvestro, L. Graham, D. Cheney, and I. Levine. 2005. Investigations of the tolerance ability of *Porphyra* to survive extreme environmental stresses. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 21.

Levine, L.A., A. Legee, Z. Pei, and D. Kong 2006. *Porphyra* osmoregulation and the isolation of a Ca^{2+} sensing receptor. 54th Annual Meeting British Phycological Society. Plymouth, UK. Book of Abstracts: 17.

Levine, L. A., A. Legee, Z. Pei, D. Kong, D. Cheney, and T. Hogan 2006. Osmoregulation in marine algae and the bioremediation of freshwater effluents. International Conference on Applied Phycology; Algae in Biotechnology and Environment. Delhi, India: Book of Abstracts: 8-9.

QPX in Hard Clams

Smolowitz R. 2005. Presentation on QPX at the Barnstable County QPX aquaculture meetings. (One in Wellfleet, MA and one in Barnstable, MA.)

Smolowitz, R. 2006. QPX investigations in Massachusetts. Presentation at the National Shellfish Conference. Monterey, CA.

Ornamental Fish

O'Shea, Stephen K., Nancy E. Breen, Bradford D. Bourque, Amanda White, Kevin Jackson, Loong Fat Ho, and Harold F. Pomeroy. 2006. A more efficient method of fatty acid analysis and the determination of carotenoid content in the study of brood stock and juvenile nutrition in marine ornamental fishes. Presented at COMOS; The Society for Small Molecule Science. Las Vegas, NV

Pomeroy, Harold F., Bradford D. Bourque, Joseph Evans, Loong Fat Ho, Amanda White, Spencer Gowan, Timothy Arcand, and Kevin Jackson 2006. Production technology and economic feasibility of marine ornamental aquaculture. Presentation at the Northeast Regional Aquaculture Conference and Exposition. Mystic, Connecticut.

Eastern Oysters

Roberts, S. B., 2006. Genomic approaches in characterizing shellfish disease: interrelationships between animal, human and ecosystem health. Cummings School of Veterinary Medicine at Tufts University, Annual Symposium: Marine and Aquatic Medicine & Conservation. North Grafton, MA. April 22.

Diner, E. , R. Smolowitz, M. Gomez-Chiarri, K. Tammi, D. Leavitt, and S. Roberts, 2006. Assessing disease tolerance in the eastern oyster using gene expression profiling. 26 th Annual NOAA-NMFS Milford Aquaculture Symposium. Meridan, CT. February 28.

APPENDICES

1. **Completion/Termination Reports for Projects**

2. **Progress Reports for Ongoing Projects**

TERMINATION REPORT

Project Code: 03-17

Subcontract/Account No. 556808
supported by 99-38500-7885 and 557203
supported by 2002-38500-12056

Project Title: "Development of diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus"

Reporting Period: November 2003 – October 2006

Funding Level: $124,612

Participants:

Steven Roberts, Project Coordinator, Marine Biological Laboratory, Woods Hole, MA
Frederick Goetz, Senior Scientist, Great Lakes WATER Research Institute, Milwaukee, WI
Scott Lindell, Aquaculture Research Specialist, Marine Biological Laboratory, Woods Hole, MA
Stewart Johnson, Associate Research Officer, National Research Council, Halifax, Nova Scotia, Canada
Deborah Bouchard, President, Micro Technologies, Inc., 41 Main Street, Richmond, ME
George Nardi, Director, GreatBay Aquaculture, LLC, 153 Gosling Road, Portsmouth, NH
David Berlinsky, Assistant Professor, University of New Hampshire, 46 College Road, Durham, NH
Nick Brown, Manager of Operations, University of Maine, 33 Salmon Farm Road, Franklin, ME, 04634
Larry Buckley, Director URI/NOAA CMER Program, University of Rhode Island, Narragansett, RI
Dale Leavitt, Regional Aquaculture Specialist, Roger Williams University, One Old Ferry Road, Bristol, RI
Chris Bartlett, Extension Associate, Maine Sea Grant College Program, 16 Deep Cove Road, Eastport, ME

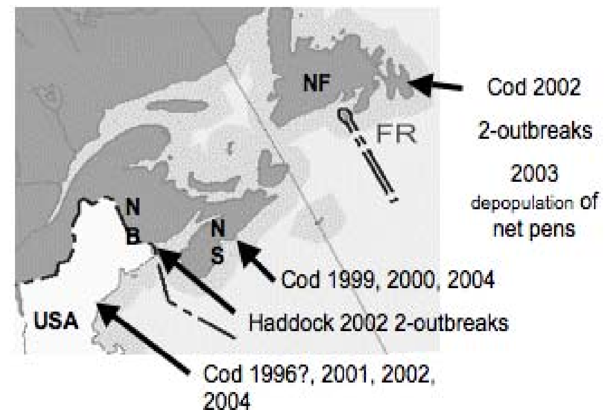
Reason for Termination: End of Project

Project Objectives:

1. Continue the development and refinement of a Real-time PCR assay for the detection of cod nodavirus in cod broodstocks, gametes, fertilized eggs and larvae.
2. Validate the use of the Real-time PCR assay for detecting nodavirus in infected tissues (brain, eyes, eggs, larvae and juveniles) and for the non-lethal detection of nodavirus in cod broodstock (via blood and/or gametes).
3. Screen captive broodstocks and progeny in New England to test the Real-time PCR assay in an industry setting and explore the development of a reagent kit.
4. Develop egg disinfection methods that effectively eliminate nodavirus on the surface of eggs and possible vertical transmission.
5. Develop extension and outreach materials and presentations for nodavirus detection and disinfection.

Background

In the last ten years, nodavirus infections that cause viral encephalopathy and retinopathy (VER, and also sometimes known as viral nervous necrosis (VNN)) have become a major disease concern for many cultured marine fish species. Nodaviruses are small (25-34 nm) icosahedral viruses that infect the central nervous system (CNS) of the fish. The genome of this virus is composed of two single stranded, positive-sense RNA molecules, RNA1 and RNA2. Outbreaks of cod nodavirus in Atlantic Canada and New Hampshire have caused catastrophic mortality at the hatchery and nursery stages (Figure 1). In order to prevent additional set-backs to New England's developing marine fish aquaculture industry, the current research project focused on obtaining a better understanding of this epidemic and means to properly manage it.



Anticipated Benefits

One of the primary benefits of the completed research is a real-time PCR assay for nodavirus that can be used to accurately quantify levels of the deadly virus. In addition, the assay can be used to detect nodavirus through non-lethal means. The diagnostic activities carried out as part of this project in combination with our egg disinfection trials contributed to the understanding that nodavirus is prevalent in the environment. The products developed as part or this project will lay the groundwork for future projects characterizing nodavirus etiology and exploring vaccine development.

Principal Accomplishments

Development of a real-time quantitative RT-PCR assay for nodavirus in cod was completed during the first year of the project. Real-time PCR monitors in actual time the fluorescence emitted during the PCR reaction as an indicator of amplicon production. In Real-time, the amplicon is produced by a pair of unlabelled primers. Interior to these primers is a dual-labeled oligonucleotide probe that is 20-30 bp with a Tm value of 10 ^{o}C higher than the adjacent primers. The probe contains a fluorescent on the 5' base, and a quenching dye on the 3' base. When the polymerase replicates a template on which a probe is bound, its 5' exonuclease activity cleaves the probe emitting fluorescence that increases in each cycle proportional to the rate of probe cleavage. Accumulation of the amplicon is then detected automatically by monitoring the increase in fluorescence of the reporter dye. Using retinal tissue from a nodavirus infected cod (provided from Stewart Johnson; Institute for Marine Bioscience, National Research Council, Canada) this assay has been rigorously validated. The technical details of the assay can be found below in Part II.

The sensitivity of this molecular assay facilitated the first non-lethal detection of Atlantic cod nodavirus. As nodavirus targets the central nervous system, customarily the brain is removed for diagnostic testing. During a nodavirus outbreak at a regional hatchery we were able to sample blood and detect circulating nodavirus. Water samples were also analyzed using the assay and nodavirus was detected. These data indicate that assay developed as part of this project would be a valuable tool to understand the nodavirus's life history and environmental factors influencing outbreaks.

Working with aquaculture industry was a major focus of the project and we worked with several commercial and non-commercial facilities (Figure 2). While most of the samples were from Atlantic cod, we did spend time on assays for nodavirus effecting flounder, haddock, pompano, and barramundi. Over 250 individual Atlantic cod samples were tested for nodavirus. Most of the samples were from eggs (62), blood (39), brains (36), and whole larvae (27). Other samples included gonads, livers, and milt. As part of the diagnostic activities, an experiment was set up to determine if Atlantic cod nodavirus could infect haddock juveniles. Following one month of exposure from preserved cod larvae severely infected with nodavirus, no haddock tested positive. Water samples taken from the exposure tank were positive for Atlantic cod nodavirus. One of the most significant impacts of the assay development was accurately diagnosing the samples from a commercial hatchery to be free from nodavirus, which saved valuable broodstock from being unnecessarily destroyed (see next section: Impacts)

.

Figure 2. Map showing the hatcheries and facilitates that provided samples for diagnostic analysis.

Egg disinfection technology was another component of the grant. Several agents were used as disinfectants on cod eggs from captive cod. Some eggs were obtained from experimentally infected broodstock. Iodine was one agent used with no effect on survival of the treatment concentration at any of the treatment times tested (up to 20 minutes at 100ppm). Ozone treatment also had no effect on survival (2ppm up to 4 minutes). A novel disinfection agent, RNAse A, used during our research.

RNAses are enzymes that specifically degrade RNA. RNAse disinfection trials were carried out (0.1ppm and 1.0 ppm for 1 minute) without negative effects on survival. No cod eggs were positive for nodavirus. It is not known whether nodavirus can be consistently transmitted from infected broodstock to the offspring. It was determined that Atlantic cod eggs can withstand high levels of disinfection and the ozone levels used were sufficient to kill nodavirus based on research by other groups. The figure at the bottom of the page shows an example of the disinfection trial set-up.
While not associated with the original objectives of this project, limited effort was placed into exploring commercial use of a vaccine. Specifically recombinant protein was produced that corresponds to the coat protein of the cod nodavirus. This protein was produced in a bacterial system (pBAD; Invitrogen) and purified using column chromatography (Figure 3). The long-term goal of such an effort would be to expose cod larvae to this synthetic protein in the hopes that their immune system would be better prepared to handle a nodavirus.

Outreach and extension has been integral to the success of this project. This involved regular communication with hatcheries and production facilities to determine the status of any outbreaks and their need for diagnostics. Regular communication with GBA, allowed us to be present during one such outbreak when we were able to obtain a comprehensive sample set.

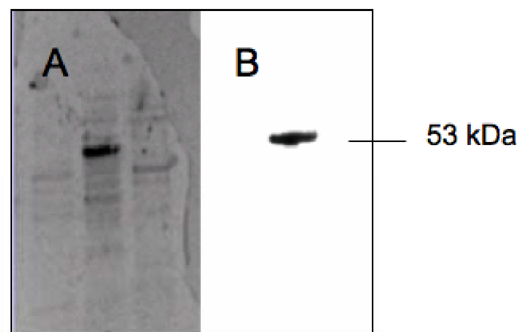


Figure 3. Images of Coomassie stained gel (A) and Western antibody was used for western analysis, corre blot (B) of recombinant nodavirus protein. An AntiV5 antibody was used for western analysis, corresponding to a V5 epitope incorporated into the construct.

Providing information to the public has also been very important. In order to effectively carry out this goal a website was developed (http://www.mbl.edu/aquaculture/nrac/) for technology transfer and dissemination of techniques for disease diagnostics and egg disinfection methods. In December 2004 our research was presented at the Nodavirus II workshop in Portsmouth, New Hampshire. The proceedings of this workshop, including presentations, are also available on site. The website also contains several presentations and technical reports. The current termination report will be made available on the website which will be maintained into the future. There has been a significant international and regional feedback as a result of the website.

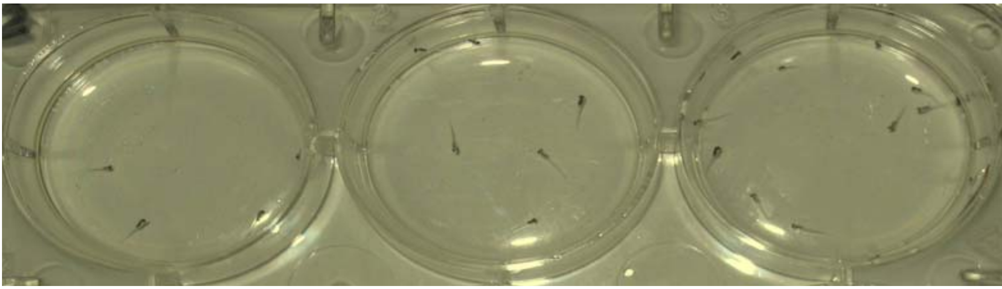


Figure 4. Portion of multi-well plate used in egg disinfection trials. This example shows cod larvae that have recently hatched. Shortly after hatching, survival data is recorded and samples are taken to assess the presence of nodavirus.

Impacts

One of the most significant impacts is our testing of cod fingerlings stocked into sea cages. During a routine health inspection at GBA (Director: George Nardi – participant on current grant) one of the sample pools came up positive for nodavirus. Upon retesting, no positives were registered by GBA's contractor. Samples were also sent out to us (Marine Biological Laboratory) for testing using the Real-time PCR assay and registered negative. Authorities used the results from the Real-time PCR assay and GBA's contract lab in the decision making process to continue to allow transfer of these fish to other sites. GBA has stated that as long as they can afford it, (i.e. through grant support) they will always try to use multiple labs and split samples to ensure accurate results. Without the assay results we provided to managers, a large number of fish might have been destroyed based on the belief that these fish had become infected with nodavirus.

Recommended follow-up activities

As mentioned above, there are two general activities that are recommended to follow-up the current project. These include characterizing etiology of Atlantic cod nodavirus in the environment to have better understanding of factors influencing outbreaks. This could include using the assay developed as part of this research as a tool to evaluate controlled and natural environments. Another recommended activity would be to explore the use of a commercial vaccine. One thing that we did learn from the research is that nodavirus is more widely present than first thought and vertical transmission of the virus is a significant concern. If a vaccine could offer any protection to the virus, this would significantly reduce the risk associated with an outbreak.

Support

	NRAC-USDA Funding	Matching Support (MBL)	Total Support
Year 1	$ 63,017	$ 7,063	$ 70,080
Year 2	$ 61,549	$ 7,063	$ 68,658
TOTAL	$124,612	$14,126	$138,738

Publications, manuscripts, or paper presented

Roberts, and Rick Goetz "*Quantitative real-time molecular methods for disease detection in marine fish"* AQUACULTURE 2004, Honolulu, Hawaii March 1-5 2004

Roberts, Steven and Scott Lindell "*Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus*" NACE 2004, Manchester, New Hampshire, December 3, 2004

Lindell, Scott and Steven Roberts "*Egg Disinfection and diagnostic techniques for Atlantic cod nodavirus*" Nodavirus II Workshop, Portsmouth, New Hampshire, December 8, 2004

Steven Roberts "*Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus*" NRAC Annual Aquaculture Research, Extension and Business Opportunities Update, Dartmouth, Massachusetts, February 24, 2005

Steven Roberts (2005) New molecular techniques to detect nodaviruses in Atlantic cod hatcheries, Global Aquaculture Advocate, April 2005, 2pp.

PART II – Technical Report

Technical Summary

One of the primary long-term goals of our research was to develop specific and sensitive viral detection techniques. In order to develop a nodavirus detection assay, we have focused on Real-time PCR technology. The specific target of our assay was the gene coding for the coat protein of the virus (RNA2: GenBank Accession #AF445800). A dual-labeled probe was designed, NVcap (FAM- CAT CCC TTG AGA CGC CCG AAC - BHQ1) that is flanked by two primers, NVtmf (TCG CTG GAG TGT ACG TCT CAG T) and NVtmr (GAG TGG TCC GAG GGT TAG GAT). The assay is performed using Invitrogen's Thermoscript One-Step RT-PCR kit with an Opticon Continuous Fluorescence Detection System (MJ Research). Several other comparable instruments (n>10) could also be used to carry out the assay from other manufacturers. For all samples diagnosed as part of this research, Tri-Reagent (MRC Inc.) was used from RNA extraction.

The RNA extraction protocol includes the following basic steps;

1. Homogenization: 1 ml TRI REAGENT + 50-100 mg tissue
2. Phase separation: homogenate 0.2 ml chloroform.
3. RNA precipitation: aqueous phase + 0.5 ml isopropanol.
4. RNA wash: 1 ml 75% ethanol.
5 RNA solubilization: water.

Detailed instructions on RNA extraction can be found in the manufacturer's protocol (http://www.mrcgene.com/tri.htm). When water samples were tested, water was first filtered using a 0.22 um PES filter. The filter was then treated in a similar manner as tissue. A schematic outlining the steps required to complete the molecular assay is shown on the following page. We did explore the possibility of developing a reagent kit, however given the rapid improvement in commercial reagents and decrease in reagents costs this approach is not practical.

The initial cDNA synthesis and two-step PCR cycling program (40 cycles) is performed consecutively in the same reaction well by incubating samples first at 50 ^{o}C for 30 min, followed by PCR. For PCR, an initial 5-min 94 ^{o}C incubation is performed followed be 40 cycles of denaturation (94 ^{o}C for 15s) and annealing/extension (60 ^{o}C for 1 min). Fluorescent detection is performed after each annealing/extension step. To determine the lower detection levels of the molecular assay and dynamic range, nodavirus RNA was generarted *in vitro*. In other words, synthetic RNA was generated that can be directly correlated with nodavirus quantity. Specifically, cod nodavirus RNA was transcribed *in vitro* using a T7 polymerase and the RiboMAX Large Scale Production System (Promega).

1: AY847947. Reports Atlantic cod nerv...[gi:57115615]

Features Sequence

```
LOCUS       AY847947                 670 bp    RNA     linear   VRL 10-JAN-2005
DEFINITION  Atlantic cod nervous necrosis virus strain DB5y5_85 putative RNA
            dependent RNA polymerase gene, partial cds.
ACCESSION   AY847947
VERSION     AY847947.1  GI:57115615
KEYWORDS    .
SOURCE      Atlantic cod nervous necrosis virus
  ORGANISM  Atlantic cod nervous necrosis virus
            Viruses; ssRNA positive-strand viruses, no DNA stage; Nodaviridae;
            Betanodavirus.
REFERENCE   1  (bases 1 to 670)
  AUTHORS   Roberts,S.B.
  TITLE     Molecular detection of Atlantic cod nodavirus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 670)
  AUTHORS   Roberts,S.B.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-DEC-2004) Marine Biological Laboratory, 7 MBL Street,
            Woods Hole, MA 02543, USA
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Figure 5. NCBI Genbank entry for nodavirus RNA polymerase gene.

In order to explore additional targets for molecular diagnostics the other portion of the Atlantic cod nodavirus was isolated. This was completed using a degenerative PCR based approach. This nucleotide was information was immediately made available to the public through the National Center for Biotechnology Information so other researchers could use it to study the organism (Figure 5). Our molecular assays showed that target this nucleotide sequence was not superior to targeting the capsid protein.

Another objective of our research was to screen other species from regional hatcheries. We have evaluated samples for several research and commercial based production facilities (Figure 2). To accommodate the request to test species other than cod, we developed an assay that is similar to the dual labeled probe assay for cod described above, however instead of the cleavage of a dual-labeled probe emitting fluorescence, SYBR green I dye is included in the reaction that in turn, fluoresces when it binds to the final product (double-stranded DNA). For this assay we used Stratagene's Brilliant SYBR Green Master Mix kit. The primers that proved to be the most successful for this assay were:

```
Funiv  TCT TCC AGC GAT ACG CTG TTG A
Runiv  TCA GTG TTG TTG CCG RCA CAC A
```

Numerous egg disinfection trials were conducted as part of this research. As summarized in the previous section three agents were used; iodine, ozone, and RNase A. Trials were carried out in triplicate using 5cm plastic Petri dishes (Figure 4). Iodine disinfection was carried out at 100ppm for 2, 10, and 20 minutes. Ozone treatments were carried out at 2 ppm for 1, 2, and for minutes. The levels for the first two treatments were based on levels commonly used by the industry. To our knowledge, RNase A had never been used as an egg disinfection agent two doses were selected based on the amount of RNA reported to be degraded by one unit of enzyme. The two doses included 0.1 ppm and 1.0 ppm, both for a duration of 1 minute. Neither of these treatments appeared to significantly effect survival. The table below shows the specific survival rates for iodine and ozone treatments.

Treatment	**Exposure Time**	**Survival (7 d post hatch)**
Iodine (100ppm)	2 min	93%
Iodine (100ppm)	10 min	92%
Iodine (100ppm)	20 min	81%
None (Iodine control)	na	90%
Ozone (2ppm)	1 min	96%
Ozone (2ppm)	2 min	90%
Ozone (2ppm)	4 min	98%
None (Ozone control)	na	88%

TERMINATION REPORT

PROJECT CODE: 03-1 SUBCONTRACT/ACCOUNT NO: 557005

PROJECT TITLE: "Development of Culture Methods for Commercial Production of Rainbow Smelt (*Osmerus mordax*)"

DATES OF WORK: 1/1/03-12/30/05

PARTICIPANTS: Principle Investigator(s):
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Dr. James Haney, Professor & Chair, Dept. of Zoology, UNH, Durham, NH 03824
Phone 603-862-4814, FAX 603-862-3784, email JFHaney@hypatia.unh.edu
Ms. Joyce Newman, Aquaculture Extension Specialist, Cooperative Extension, UNH, Durham, NH 03824 Phone 603-749-1565, FAX 603-743-3997, email jj.newman@unh.edu
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Mr. John Whalen, Owner/Operator Harmon Brook Farm, PO Box 373, Canaan, ME 04924
Phone 207-474-1215, FAX 201-858-0366, email hbfarm@kynd.com
Non-funded Collaborators:
Dick Wollmar, Owner/Operator, Moor Farm, 109 Walnut Avenue, North Hampton, NH 03862
Phone 603-964-6793
Joseph Vaillancourt, Owner/Operator, Garrison House Farm, 151 Packer's Falls Road, Durham,NH 03824 Phone 603-659-3577

REASON FOR TERMINATION: Objectives completed

PROJECT OBJECTIVES:

1) Optimize environmental conditions for intensive production of rainbow smelt larvae.
2) Investigate pond culture methodologies that promote high survival and growth of rainbow smelt larvae
3) Determine the optimal feeding progression of prey species conducive for larval smelt survival
4) Develop a practical protocol for rearing rainbow smelt larvae for commercial production
5) Teach established and prospective aquaculturists techniques for profitable smelt culture for development of a new industry

ANTICIPATED BENEFITS:

The goal of this project is to develop specific culture methods for rainbow smelt in order to help establish an industry centered in the Northeast. Commercial culture of rainbow smelt would: 1) reduce fishing pressures on wild stocks, 2) provide economic opportunities for established and novice culturists and 3) increase the available supply of rainbow smelt to bait dealers to meet existing demand. In addition, techniques developed for culture of rainbow smelt will likely be transferable to other species, including the threatened delta smelt (*Hypomesus transpacificus*).

PRINCIPLE ACCOMPLISHMENTS

Objective 1- Optimize environmental conditions for intensive production of rainbow smelt larvae.

Complete details of the findings for this objective are presented in Part II, Technical Analysis and Summary. Broodstock were collected by fyke net from the Exeter River during their annual spawning migrations in March 2003-05. The fish were transported to the University of New Hampshire and strip-spawned, without hormone induction, within 5 d of capture according to our previously published methods (Ayer et al. 2005). To determine the effects of salinity and temperature on hatching success, fertilized eggs were cultured in sterile Petrie dishes under controlled conditions. Eggs were incubated at 5, 10, 15 and 20 C at salinity levels of 0, 10, 20 and 30 ppt. For larval experiments, the embryos were cultured in 2 l MacDonalds's jars until hatching occurred. Upon hatching, larvae were assigned to experiments to assess the effects of salinity, temperature, addition of microalgae and light intensity (100 vs. 500 lx) on growth and survival.

Our data indicated that hatching occurs over a range of temperatures from 5-20° C but was greatest at 10 and 15 °C. No hatching occurred at 20 or 30 ppt but was not different at 0 or 10 ppt. Larval survival was significantly impacted without the addition of microalgae to the culture water (87% survival vs. 17%) but not by light intensity. Growth was greater at 20 vs. 15 °C and declined with higher salinity concentrations (20 < 10 < 2 ppt and 8 < 5 < 2 ppt).

Objective 2. Investigate pond culture methodologies that promote high survival and growth of rainbow smelt larvae.

In April-June, 2003-05 broodstock were harvested from the Kennebec River, transported to Harmon Brook Farm (Canaan Maine) and spawned as above. On average, twenty thousand larvae (5 DPH) were stocked in 6-0.2 hectare ponds that had been enriched for zooplankton growth and rid of predatory insects. Survival of smelt was extremely variable among ponds ranging from 0 to approximately 2000. The reason for the wide variability was likely due to prey availability. Upon harvest in the fall (Oct-November) smelt were 60-110 mm in length.

Objective 3. Determine the optimal feeding progression of prey species conducive for larval smelt survival.

In the laboratory, we determined that at 10-15°C larval smelt begin feeding on marine rotifers (*Brachionus plicatilis*) at ~3 days post-hatch (DPH) and will accept *Artemia* nauplii beginning approximately 28 DPH. At 60 DPH larvae began accepting commercially prepared diets (400 µm). In pond culture, the smelt larvae primary preyed upon freshwater rotifers at the start followed by daphnia. Consumption of commercial prepared diet variable.

Objective 4. Develop a practical protocol for rearing rainbow smelt larvae for commercial production.

We developed methodologies for spawning wild-caught smelt with extremely high levels of fertilization (>95%) and hatch (>80%). This accomplishment was made possible primarily by eliminating bacterial and fungal infections that plagued earlier attempts by removing the adhesiveness of the eggs prior to incubation. This step permitted frequent water changes and sufficient aeration necessary high embryonic survival. Additionally we developed culture conditions (temperature, salinity, greenwater) and feeding protocols for commercial scale production larval production.

Objective 5. Teach established and prospective aquaculturists techniques for profitable smelt culture for development of a new industry

The methodologies developed during these studies were used by our industry partner (John Whalen) for the first successful sales of farm reared smelt as bait for recreational fishing. Interest in raising smelt has been expressed, by a number of novice culturists (including our non-funded participants, Mr. Dick Wollmar and Joseph Vaillancourt, and others (e.g. Mr. Lou Newski, Berwick, ME)) through our outreach efforts. The results were presented at aquaculture meetings ("Development of practical culture methods for rainbow smelt *Osmerus mordax*"- M. Ayer and D.L. Berlinsky Northeast Aquaculture Conference and Exposition. December 4, 2004, Manchester NH) as well as in scientific journals. Ms. Julie Newman coordinated outreach activities with all interested baitfish farmers.

IMPACTS:

1) Working protocols for commercial-scale hatchery production of rainbow smelt were developed.
2) Results were presented in peer-refereed scientific publications and aquaculture meetings
3) Sales of farm-raised smelt were initiated in 2005.
4) Mr. John Whalen, our industry collaborator submitted a Small Business Innovative Research proposal to the USDA, to further commercialization of smelt culture
5) Additional growers are initiating smelt culture

RECOMMENDED FOLLOW-UP ACTIVITIES

Future studies should explore nutritional needs of juvenile smelt through the growout phase as well as harvesting and transport methodologies.

SUPPORT:

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVERSITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
1	$126,208						$126,208
TOTAL	$126,208						$126,208

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Ayer, M.H., C. Benton, W. King V, J..Kneebone, S. Elzey, M. Toran, K. Grange and D.L. Berlinsky. Development of Practical Culture Methods for Rainbow Smelt Osmerus mordax Larvae. North American Journal of Aquaculture. 2005. 67:202-209.

DeGraaf, J.D., and D.L. Berlinsky. Cryogenic and refrigerated storage of Rainbow smelt (Osmerus mordax) spermatozoa 2004. Journal of the World Aquaculture Society. 35 :(2) 244-231.

TERMINATION REPORT - PART I

PROJECT CODE: 03-14

SUBCONTRACT/ACCOUNT NO: 556805 (Grant # 99-38500-7885)
556908 (Grant # 00-38500-8990)

PROJECT TITLE: **Salmon Hatchery Effluent Management Utilizing Integrated Polyculture Technologies**

REPORTING PERIOD: January 1, 2004 – August 1, 2006

FUNDING LEVEL: $ 150,000

PARTICIPANTS: Ira Levine, Donald Cheney, Rebecca Lebrun, Tony Legee, Bobbi Cooke, Hitoshi Kito, Dongdong Kong, Zhing Pei

REASONS FOR TERMINATION:

Indicated objectives completed

PROJECT OBJECTIVES:

1. Development of a "Zero-salinity" or freshwater tolerant, fast-growing strains of *Porphyra yezoensis*.

2. Determination of optimal *Porphyra yezoensis* cultivation strategies for N and P removal.

3. Identification of molecular aspects of salinity tolerance.

4. Technical transfer of project results.

ANTICIPATED BENEFITS:

Benefits include the newly developed euryhaline cultivars capable of producing r-phycoerythrin in freshwater aquaculture systems. Determination of N and P uptake dynamics, and the understanding of the molecular aspects of salinity balance in *Porphyra*. The competitive advantage of integrated polyculture will assist the land-based finfish industry in competing with global commercial interests, reduction of finfish effluents, and development of a second cash crop.

PRINCIPAL ACCOMPLISHMENTS:

1. Development of *Porphyra yezoensis* cultivar maintaining growth rates of 10 -15% per day in salinities as low as 8ppt. Additionally, cultured *Porphyra yezoensis* grown in 0.125 ppt seawater supplemented with divalent cations sustained growth rates of 3% per day.

2. Osmoregulated *Porphyra yezoensis* (supplemented with divalent ion cocktail) absorbed 16.6 – 23% of the freshwater trout effluent available nitrogen-nitrate with and without nutrient enrichment.

3. The isolation and sequencing of the putative *Porphyra* CAS.

4. One state, one regional and two international presentations of the grant's results. Additionally, one peer reviewed article has been published.

IMPACTS:

As the salmon aquaculture industry significantly reduces production and the number of hatcheries in the State of Maine, the challenge of effluent remediation by the originally intended end-user of this research becomes moot. The development of osmoregulated algal polyculture systems will become a reality when higher growth rates are achieved or when brackish water aquaculture systems are identified.

RECOMMENDED FOLLOW-UP ACTIVITIES:

Continued funding for CaS research and the development of osmoregulated algal culture systems are necessary to advance this concept further.

SUPPORT:

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVERSITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
2004	75000	15000					$ 90,000
2005	75000	18645					$ 93,645

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Papers Presented

Levine, I., D. Cheney. 2005. *Porphyra* from sea to freshwater; osmoregulation in marine algae. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 19.

Liu, Y.C., T Hogan, A. Cary, A. Silvestro, L. Graham, D. Cheney, and I. Levine. 2005. Investigations of the tolerance ability of *Porphyra* to survive extreme environmental stresses. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 21.

Levine, I., A. Legee, Z. Pei, and D. Kong. 2006. *Porphyra* osmoregulation and the isolation of a Ca^{2+} sensing receptor. 54th Annual Meeting British Phycological Society. Plymouth, UK. Book of Abstracts: 17.

Levine, I., A. Legee, Z. Pei, D. Kong, D. Cheney, and T. Hogan. 2006. Osmoregulation in marine algae and the bioremediation of freshwater effluents. International Conference on Applied Phycology; Algae in Biotechnology and Environment. Delhi, India: Book of Abstracts: 8-9.

Publications

Levine, I. 2006. Case study on *Porphyra* cultivation in Maine, USA and New Brunswick, Canada. In: Aquaculture Compendium, Online at www.cabicompendium.org/ac. Wallingford, UK: CAB International. pp 1-13.

Termination Report - PART II

In the northeastern United States, Atlantic salmon and other finfish (trout, tilapia, halibut, cod and haddock) are the dominant aquacultured species with > 20 finfish hatcheries whose ability to meet EPA's new discharge standards is a matter of concern. The development of a practical integrated seaweed: finfish integrated aquaculture system would bioremediate hatchery effluents while producing a second commercial crop. Examples of valuable byproducts found in *Porphyra yezoensis* include: antioxidant carotenoid pigments such as beta-carotene (0.705 mg/g DW) and lutein (1.339 mg/g DW), long-chained polyunsaturated omega-3 fatty acids such as eicosapentaenoic acid (EPA; 6-12 mg per g DW), various vitamins (C, E and B12), and the pigment r-phycoerythrin C, which is utilized as a fluorescent “tag” for immunofluorescent studies.

One advantage of modifying marine algae, e.g. *Porphyra* spp. to bioremediate effluents from a freshwater fish hatchery is the ability to absorb ammonia and nitrate simultaneously at high rates, in contrast with phytoplankton and many vascular plants which often stop using nitrate as ammonia levels increase.

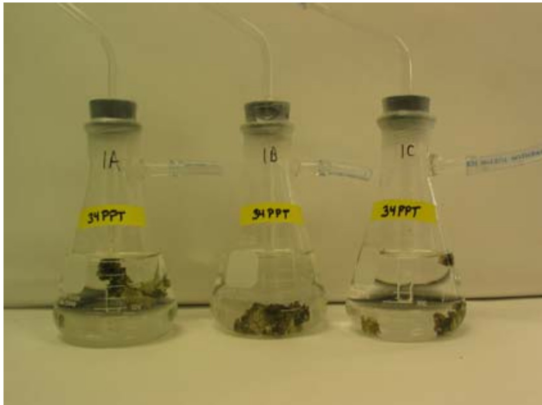
Calcium ions have been demonstrated to exert key regulatory effects on algae when exposed to low salinities. Previous reports suggest that: 1) calcium is a key element in determining algal survival at low salinities and 2) that genetic isolates of various seaweeds may possess alterations in distinct genes including Calcium Receptors (CaR) that provide them with the ability to adapt to the shifting salinities. CaRs have been shown to be the “master controller” of divalent calcium homeostasis in humans and salinity sensors in flowering plants, aquatic organisms, and green algae. Preliminary efforts have suggested similar molecules in *Porphyra*.

The development of a land-based, coastal independent system, utilizing low salinity-tolerant (via classical and emerging proprietary methodologies) strains of *Porphyra* was the focus of both cultural and molecular investigations funded through the USDA NRAC Grant 03-14.

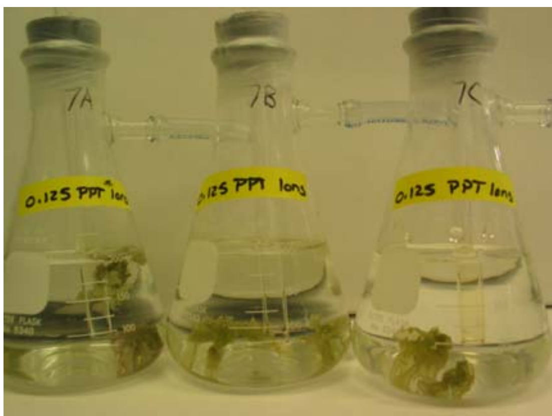
Objective 1:

Initial efforts have yielded sporophytic and gametophytic strains of *Porphyra yezoensis* that maintain 10 -15% per day growth rates in salinities as low as 8ppt. Our team has successfully cultured *Porphyra yezoensis* in 0.125 ppt seawater supplemented with divalent cations achieving limited growth rates of 3 % per day in 16 °C at reduced light levels. PI increased the scope of the year one research plan and included both the gametophyte (as originally described in the grant proposal) and the sporophyte or conchocelis form of the plant life history (Addendum I) in the low salinity experimentation.

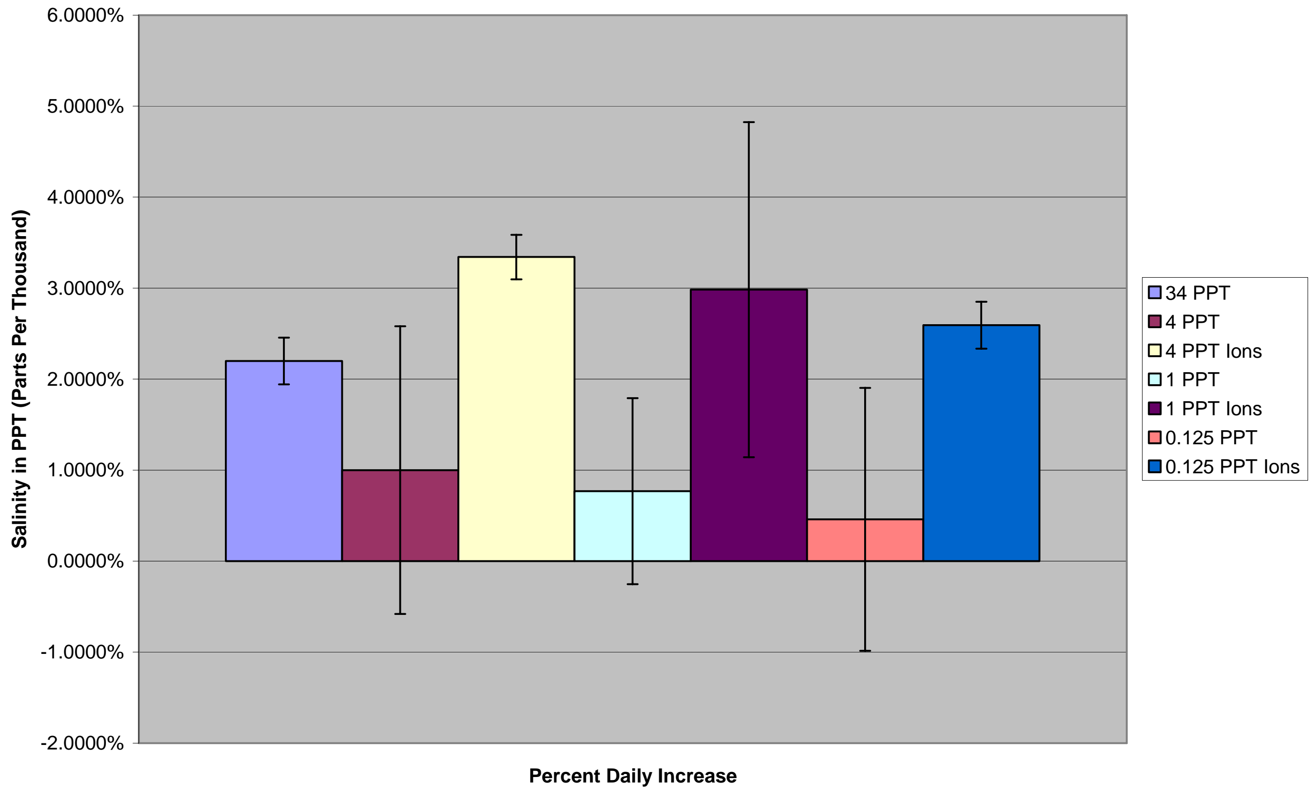
The development of a freshwater *Porphyra* cultivar, the singular goal of year one of this two-year research effort, has been achieved. Thalli grew at a greater rate although not significantly greater in ion-supplemented freshwater as compared to the control of full strength seawater.



Full Strength Seawater (32 ppt) T= 21 days



Freshwater (0.12 ppt) T = 21 days

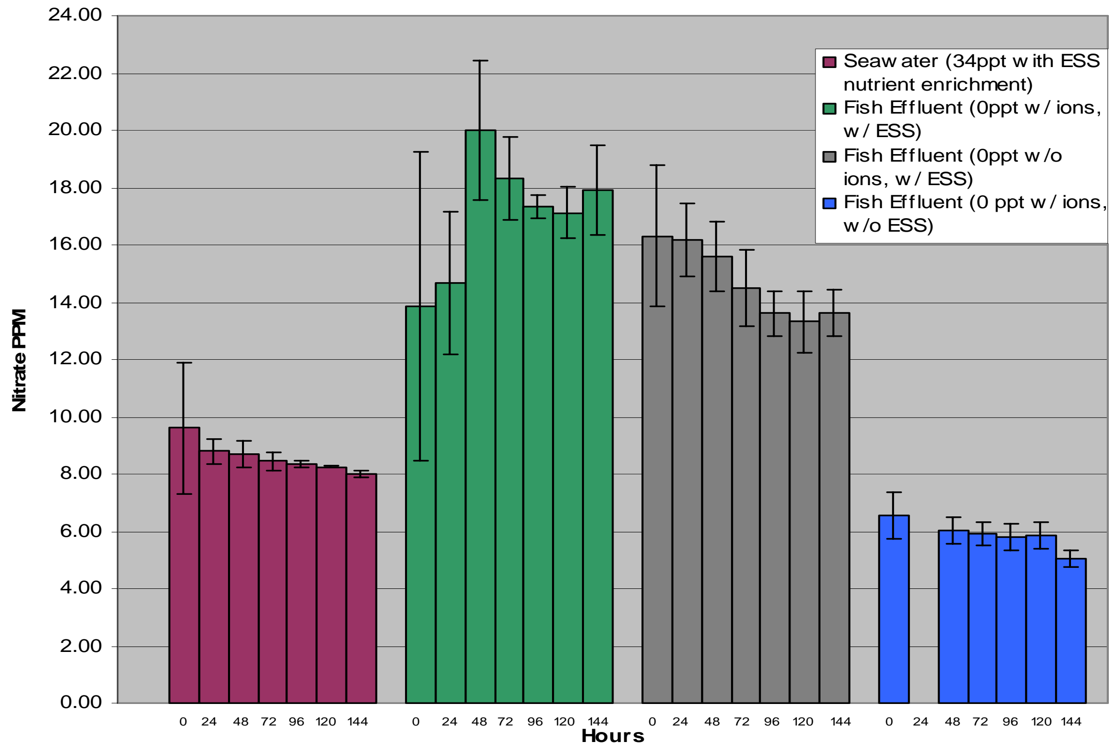


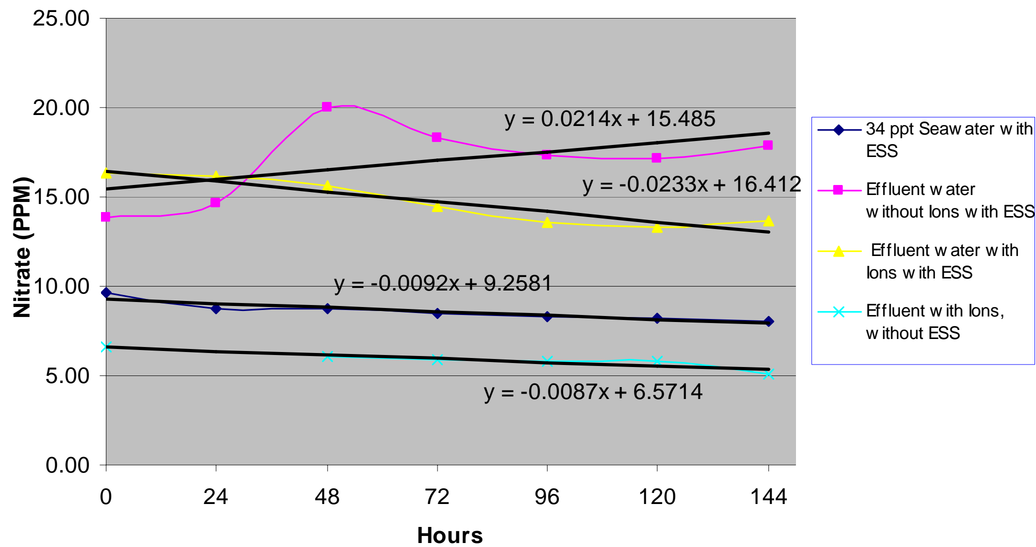
The cultivation research in months 13-18 have been significantly hindered by the cross contamination of our low salinity cultures by an unidentified cyanobacteria which increased its growth rates as a function of the decrease in salinity.

Objective 2:

Osmoregulated *Porphyra* nitrogen uptake dynamics are independent of salinity variation (0-34ppt). Osmoregulated *Porphyra yezoensis* (supplemented with divalent ion cocktail) absorbed 16.6 – 23% of the freshwater trout effluent available nitrogen-nitrate with and without nutrient enrichment, respectively as compared to 17% absorption of the full seawater control over the course of the 7-day experiment (Figure 1). The highest rate (m = -0.023) of nitrate uptake was realized in the 0ppt fish effluent with ion supplements and ESS (Figure 2); uptake was approximately 35% more rapid then that of ion supplemented fish effluent without extra nutrients added.

Over the course of the 7-day experiment, nitrate absorption was evident in all, but the non-osmoregulated, 0 ppt fish effluent without supplemental ion treatments. The non-osmoregulated thalli treatment increased in nitrate levels (+ 29%) possibly due to tissue deterioration and pigment loss observed after 48 hours. Observations of both osmoregulated treatments (with and without added ESS) and the full strength seawater control indicated substantial tissue integrity and pigment retention. Comparison of uptake rates between the osmoregulated thalli cultured in fish effluent without ESS nutrient supplementation and that of the full seawater control reveals nearly identical uptake rates (m = - 0.0087 and – 0.0092, respectively).





Objective two's research effort determined the ability of osmoregulated thalli to bioremediate fish effluents as part of a polyculture system. The data revealed nearly equal uptake rates between the control and the osmoregulated thalli. Thalli integrity and pigment concentrations were also observationally equal indicating a healthy system.

The highest rate of nitrate uptake was observed in the nutrient supplemented, osmoregulated treatment which would indicate the ability to bioremediate hyper accumulated fish effluents along with land-based, agricultural produced effluents.

Objective 3:

The development of a freshwater *Porphyra* cultivar, the singular goal of year one of the two-year research effort was achieved (see Year 1 Annual report dated November 19, 2004). As per the annual report the efforts to develop the molecular nature of salinity tolerances, the cloning of the CAS gene has made significant progress.

Results to date include the isolation and sequencing of the putative Porphyra CAS. Database analyses indicate homologues inOryza sativa, Arabidopsis thaliana, and Chlamydomonas reinhardtii. Future efforts include determination of Ca^{2+} binding capacity, and subcellular localization.

CAS gene cloned from *Porphyra yezoensis*

Part II -- Adendum I

Objectives

The main objectives of our work during the first six months of this project were to
1) Investigate the best methods for growing conchocelis of *Porphyra yezoensis*, 2) determine its salinity tolerance limits, and 3) initiate studies to produce a low-salinity tolerant strain that is faster growing than is being currently being tested.

Major Accomplishments to Date

1. **Establishment of conchocelis culture conditions**.

"Conchocelis" refers to the filamentous, diploid stage in the life cycle of a *Porphyra* species (see Figure 1). We are interested in learning how to grow conchocelis because of its very different, and potentially valuable, polyunsaturated fatty acid (PUFA) composition from that found in blades. In particular, previous work (Graham and Cheney, unpublished) has shown that the most abundant PUFA found in conchocelis of *Porphyra yezoensis* is arachidonic acid (AA), while that found in blades is eicosapentaenoic acid (EPA); both can play an important role in the early development and growth of fish and are of commercial value.

TERMINATION REPORT – Part III

PROJECT CODE: 03-14

SUBCONTRACT/ACCOUNT NO: 556805 (Grant # 99-38500-7885)
556908 (Gant #00-38500-8990)

PROJECT TITLE: Salmon Hatchery Effluent Management Utilizing Integrated Polyculture Technologies.

Although our laboratory has a great amount of experience growing the haploid blade stage of *Porphyra*, we and most labs have very little experience growing the conchocelis stage. Thus, one of our first goals was to determine the best culture conditions for growing conchocelis. However, before we could initiate conchocelis culture experiments, we had to have "clean" (i.e. contaminant free) culture material. The conchocelis material we had growing in our lab we discovered was contaminated with yeast and bacteria. We developed a simple "conchocelis decontaminating method" that kills these and other contaminants but does not harm the plants. The method consists treating the conchoecelis with a 1% solution of the commercial disinfectant Proviiodine Iodine Solution (which is a 10% iodine solution) for 60 seconds. This cleaning method has also worked on decontaminating a new conchocelis culture we received from Japan (see below).

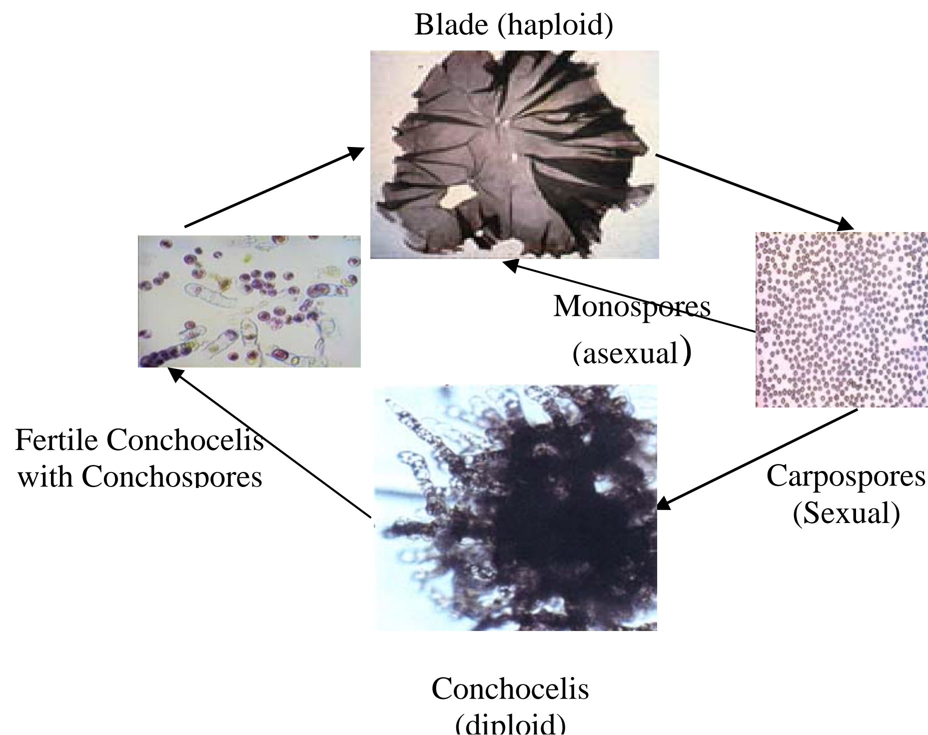


Fig. 1. Porphyra life cycle

Initial culture experiments were conducted to determine the best preliminary light and temperature conditions for growing conchocelis of two strains of *P. yezoensis* (strain #9-13, a patented strain produced by my lab over five years ago, and its parent strain #U-51) in wells. The conchocelis was subdivided into small clumps 1-2 mm in dia and grown one clump per well (see Figure 2) in a "24-well plate." Because the conchocelis in each well is too small and light to weigh, we measured growth over a 3 week period by estimating the change in clump size over time using methods similar to those used in the past to measure conchocelis growth (e.g. Waaland et al 1987; Varela-Alvarez et al 2004).

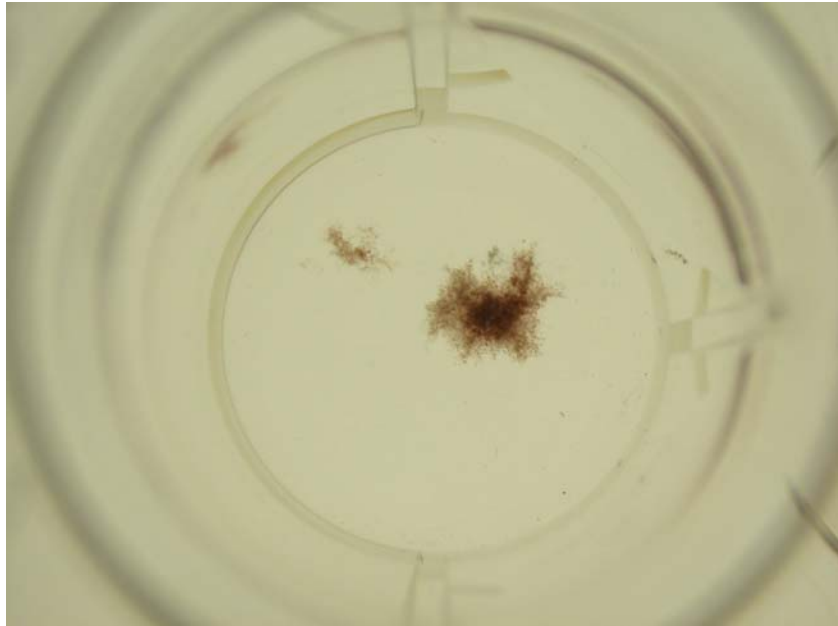


Figure 2. Conchocelis growing in a well of a “24-well plate”

Preliminary results include:

1. Both conchocelis strains could be quickly increased in biomass by subdividing it weekly and growing it in wells of a 24-well plate. After a clump was “subdivided”, we observed that the “daughter” clumps of conchocelis tend to "attach" or stick to the bottom of the well, a trait that could be useful for growing large amounts of conchocelis as a “mat” covering a surface.

2. Of the four culture conditions we initially tested (high and low temperature and light); the best growth was observed at the higher temperature (16° vs 8° C) and lower light level (ca 10 µEinstein vs. 35 µEinstein) conditions. Strain #9-13 grew faster than #U-51 (see Figure 3) at these conditions and was subsequently used for salinity tolerance experiments.

2. Initial testing of salinity tolerance.

To our knowledge, there is no information available on the salinity tolerance of any *Porphyra's* conchocelis. So far we have completed only one experiment to determine the salinity tolerance of strain #9-13 conchocelis. A second experiment is underway. In our initial experiment, we measured growth at three salinities full strength seawater (32 ppt), 20 ppt, and 8 ppt. These salinities were selected because of comparable growth data for the same salinities we had for blades. As can be seen in Figure 3, the growth rate we got at 20 ppt was almost as high as at full strength seawater (250% vs. 300% increase or 2.5X vs. 3X after three weeks), while that at 8 ppt was significantly less (150% or 1.5X after three weeks). Although these growth rates are less than what we found for blades, interestingly the pattern of salinity effects is similar to that for blades. That is, there appears to be very little difference between 32 and 20 ppt but a big difference between 32 and 8ppt. Thus, because conchocelis appears to be behaving similarly to blades, we would predict that the methods that Dr. Levine is using to grow blades at "0-salinity" should work with conchocelis. We will test this idea during the next phase of the project.

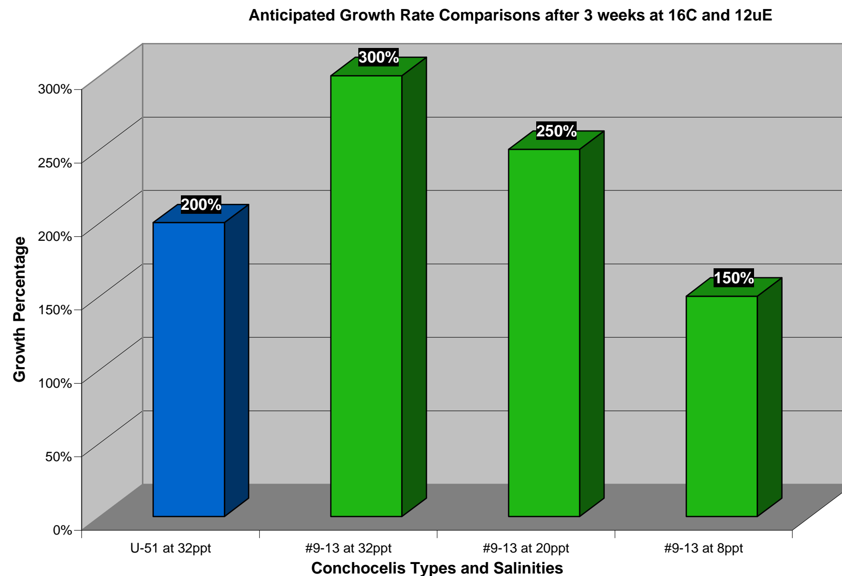


Figure 3. Growth Rate comparisons after 3 weeks at 16°C and 12 µE

3. Initial efforts to establish a "free-living" conchocelis culture system.

There are reports that *Porphyra* culturists in Japan and China are able to grow conchocelis at a fast rate in a so-called "free-living" culture system rather than a "standing" system such as in wells or the traditional oysters (Charles Yarish, personal communication). We have initiated studies to determine if we can grow strain #9-13 conchocelis in an aerated flask. So far, we have found that we can grow small fragments of conchocelis in bubblers, but only at low light levels. That is, fragments grown at higher light levels (i.e. above 12 µEinstein) become reproductive and start to produce blades, whereas conchocelis grown at light levels below 6 µEinstein remained non-reproductive. We think this may be a very promising approach and needs more experimentation.

4. Culture of new "fresh water" strains from Japan

We received two new *Porphyra* conchocelis cultures from Dr. Levine that are purported to be "fresh water" strains. These include : *P. tenera*, collected 4/9/04 from river mouth of Nagata river in Shimonoseki, and *P. katadae*, collected 2/17/04 from river mouth area of Kawatana river in Toyoura-gun, Japan. The *P. tenera* culture we received was contaminated with blue-green algae and had to be decontaminated using our "conchocelis-cleaning method" described above.

TERMINATION REPORT

PROJECT CODE: 03-3 **Subcontract/Account no: 557004**

PROJECT TITLE: Production and Culture Performance of Triploid Bay Scallops
DATES OF WORK: January 1, 2003 – April 30th, 2005

PARTICIPANTS: Funded collaborators - Dale Leavitt, Robert Rheault, Amandine Surier, Kim Tetrault, Bethany Walton, Richard C. Karney, Diane Leonard, Jack Blake, Scott Castro, Tom Berry, Roy Scheffer, Andrew Beaumont, Eileen Milanette, Max Maza.
Non-funded collaborators - Blake Barton, Rick Goetz, Ximing Guo, Jim Widman,

REASON FOR TERMINATION: All experimental goals were achieved.

PROJECT OBJECTIVES: To develop an efficient, inexpensive, and safe methodology for large-scale triploid bay scallop production. To demonstrate the culture advantages of triploids over diploids.

ANTICIPATED BENEFITS: Significantly improve the economic success of *Argopecten irradians* growers in the northeast region and to provide an option for northeast quahog and oyster farmers to diversify, therefore limiting their vulnerability to diseases and/or market shifts inherent in production centered around a limited number of culture species.

PRINCIPAL ACCOMPLISHMENTS: Superior yield of triploid bay scallops versus the diploid control was demonstrated. Close monitoring of the experimental scallops produced showed gonadal development, sign that the creation of tetraploid broodstock might be possible. Gathering of the first data on triploid cell size brings some light on the gigantism theory.

IMPACTS: Raise the interest of the shellfish community in pursuing more research on yield and tetraploid production and the causes of this increased yield (investigating gigantism).

RECOMMENDED FOLLOW-UP ACTIVITIES: Develop a USDA approved method to produce 3N scallops by developing the first 4N broostock for the bay scallop *Argopecten irradians.*

SUPPORT:

		Other Support					
	NRAC	University	Industry	Other Federal	Other	Total (cost share)	**Total**
2003	52,940	1,500 (MMA) (cost-share)	4,000 (MV fish) (cost-share)		22,173 (MVSG) (cost-share)	27,673 (c-s)	80,613
2004	50,082		2,000 (MV fish) (cost-share)		22,173 (MVSG) (cost-share)	22,673 (c-s)	72,755
Total	103,022	1,500	6,000		44,346	50,346	153,368

MMA: Massachusetts Maritime Academy
MV fish: Martha’s Vineyard Fishermen/Growers(Jack Blake, Tom Berry, Scott Castro, Roy Scheffer)
MVSG: Martha’s Vineyard Shellfish Group

PUBLICATION, MANUSCRIPTS, OR PAPERS PRESENTED:

Publications in Print:

Surier. A., 2004. Improving aquaculture through triploidy global aquaculture advocate, 7-3: 62.
Zarnoch, C., **S.Hoffman**, M. Schreibman, A. Surier, and R. Karney 2005. The biochemical composition and adductor muscle cell size in diploid and triploid bay scallops, *Argopecten irradians*. Poster presented at the Brooklyn College Science Day; 17 May 2005.

Surier, A., R. Karney, C. Zarnoch, K. Tetrault, R. Rheault, Dr X. Guo, Dr Y. Wang, B. Walton 2006. Impact of triploidy on growth, shape, muscle size, biochemical composition and fiber size in the bay scallop Argopecten irradians. Poster for the National Shellfisheries Association conference 06, Monterey, California 2006.

Manuscript:

Barton, B. 2003. Comparison of growth and survival rates of diploid and triploid bay scallop (*Argopecten irradians*) larvae induced with 6-dimethylaminopurine chemical treatment. A thesis submitted in partial fulfilment of an MSc degree in Mariculture Science and Technology at North Atlantic Fisheries College.

Maza-Iglesias, M. 2004. Triploid *Argopecten irradians*: methods, biological consequences and value for the shellfish industry. A thesis submitted in partial fulfilment of an MSc degree in Shellfish Biology, Fisheries and Culture at the School of Ocean Sciences, University of Bangor, Wales, UK.

Papers Presented:

Surier, A., and **Richard C. Karney** 2004. Induced triploidy in the Bay Scallops *Argopecten irradians* and early field culture performance Presentation for World Aquaculture'04, Honolulu, Hawaii, USA.

Surier, A., and **Richard C. Karney** 2004. Induced triploidy in the Bay Scallops *Argopecten irradians* and early field culture performancepresentation for the Milford Aquaculture Conference, New Haven, Connecticut, 2004.

Surier, A., Richard C. Karney, Kim Tetrault and Robert Rheault 2004. Induced triploidy in the Bay Scallop *Argopecten irradians* and field culture performance poster for Northeast Aquaculture Conference and Exposition '04, Manchester, New Hampshire, USA.

Surier, A., Richard C. Karney, Kim Tetrault and Robert Rheault. 2005. Induced triploidy in the Bay Scallop *Argopecten irradians* and field culture performance presentation for Aquaculture America 05, New Orleans, Louisiana, US.

Surier, A., Richard C. Karney, Kim Tetrault and Robert Rheault. 2005. Induced triploidy in the Bay Scallop *Argopecten irradians* and field culture performance presentation for the Milford Aquaculture Conference, New Haven, Connecticut, US.

Surier, A., Rick Karney, Chester Zarnoch, Kim Tetrault, Robert Rheault, Dr. Ximing Guo , Dr.Yongping Wang , Bethany Walton 2006. Impact of triploidy on growth, shape, muscle size, biochemical composition and fiber size in the Bay Scallop *Argopecten irradians*" poster for National Shellfisheries Association Conference, Monterey, CA, US.

TECHNICAL ANALYSIS AND SUMMARY :

Project Year I

Getting started - spring 2003.

Broodstock scallops were successfully conditioned and a flow-through system was found to be more effective than a static water system.

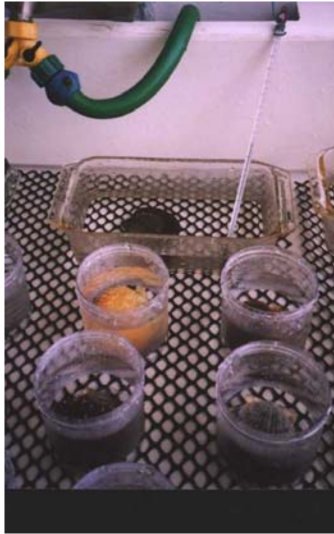
Condition Broodstock In the natural environment scallops develop their gonads each spring in response to increasing water temperatures and the presence of adequate food. In order to get an earlier start on our triploid production experiments, the broodstock were artificially conditioned to ripen earlier than their natural cycle The broodstock were conditioned by Beth Walton at the Massachusetts Maritime Academy (MMA) aquaculture facility. Starting in February, stock cultures of phytoplankton were worked up into 200 liter volumes harvested for food. On 2/14/03, 180 – 200 broodstock bay scallops from Menemsha Pond /Aquinnah were delivered to the MMA lab. Scallops were conditioned in both flow-through and static trash barrel systems.

Figures 1 and 2. Broodstock setup at the Massachusetts Maritime Academy aquaculture facility – Feb. 03

In the barrel system, scallops were alternated daily to a clean barrel with new food and heated water. We noted spawning in one of the barrels on 3/25/03, probably the result of the handling inherent in this type of conditioning system. Further, the scallops did not ripen as well as those in semi-flow through system. This system was shut down in early May and animals were combined with the others in the semi flow-though system trays. The semi flow-through system involved a trickle flow of 18°C seawater through a heated reservoir to 30 – 40 scallops held on three tiers. The animals were fed daily by replenishing each of 3 (one per tank) 20 L drip buckets. Late afternoon or evening, batches of food were also added to the flow through system as necessary. Bay scallops typically take 4 – 6 weeks to become ripe out of season. Ours took about 8 weeks.

For the first spawn on April 7 2003, the male part of the gonads seemed ripe but the female part were not and the animals did not produce any eggs. After increasing the amount of *Chaetoceros* fed, the gonads looked more plump and were full of both gametes for the April 22nd spawning.

The bay scallops appeared to become stressed after the two spawning attempts and we began to see some mortality. Conditioning ended on 5/23/03, when 35 animals were sent to Martha's Vineyard for a third spawning attempt.



Figures 3 and 4. Spawning bay scallops for triploidy induction – the scallops are placed in individual dishes for a better control of fertilization.

For the fourth and fifth spawn, naturally ripened broodstock were harvested from Lagoon Pond in Oak Bluffs, and Menemsha Pond in Aquinnah.

Eggs free of sperm contamination, essential for triploid induction, were successfully collected. Several possible methods of sperm inactivation were suggested and one method was tested.

Spawning - Synchrony of eggs is essential to triploidy induction. It is crucial that the eggs are kept free of sperm during spawning and that fertilization of all eggs occurs at the same time. The bay scallop is a functional hermaphrodite that sheds sperm and eggs intermittently during the same spawning. This condition posed a special challenge for the project.

In some species, eggs and sperm can be collected separately by "stripping", i.e., surgically extracting gametes from the gonad. In bay scallops functional gametes cannot be collected in this way but can only be gathered through a natural spawning event resulting from heat shock stimulus.

The ripe broodstock were placed in Pyrex dishes filled with filtered seawater. A fresh water bath was used to bring the temperature up to 30-32ºC and cooled back to ambient temperature. As soon as eggs were released, they were collected and inspected for sperm contamination. If sperm or polar bodies were observed (sign of early development) the eggs were not used.

When eggs were released first, they were usually free of sperm and could easily be isolated in a container for future fertilization prior to triploidy induction. In most cases, if the scallop released sperm first, despite rinsing the animals to remove excess sperm, an unacceptable level of sperm contamination could not be avoided.

Realizing that the collection of sperm free eggs would be a major impediment, we solicited the assistance of Dr. Goetz from the Marine Biological Laboratory in Woods Hole for possible ways to inhibit sperm activity. Dr. Goetz suggested several possible methods including calcium free seawater and soybean trypsin inhibitor. Dr. Goetz and Beth Walton attempted to test these compounds in early March but were unsuccessful as the scallops were not yet ripe.

On May 23rd another spawn was organized at the MV shellfish hatchery to test the efficiency of one of the compounds: calcium free seawater. The results showed that the scallops could not tolerate immersion in the calcium free seawater. Eggs that were held in calcium free seawater until fertilization resulted in 75% deformity. Luckily, a sufficient number of females shed their eggs first during four attempts allowing us to proceed with the treatments and produce triploid bay scallops.

Figures 5 and 6. The eggs were immersed in a 400 µM solution of 6-DMAP then rinsed with sea water.

Triploidy inductions were conducted at the MVSG hatchery on the 4/7/03, 4/22/03, 5/23/03, 6/28/03 and 7/31/03. On April 22nd Andy Beaumont of the School of Ocean Science, Wales, a consultant to the project, was on hand to assist and provide technical expertise.

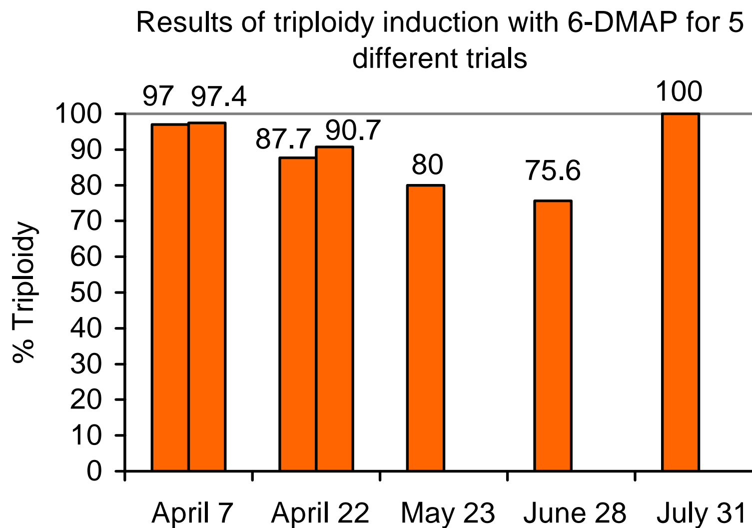


Figure 7. Results of triploidy induction with 6-DMAP summer 2003.

For each attempt the chemical treatment involved placing the eggs into a 400 µM solution of 6-DMAP 15 to 25 minutes after fertilization. After 11 minutes the eggs were rinsed with seawater and transferred into larval culture conicals. A diploid control was simultaneously cultured for each spawn.

Success of induction was assessed by flow cytometry at the Virginia Institute of Marine Science.

Figure 7 shows the various results obtained when inducing triploidy on 5 different occasions throughout the summer season 2003.

Triploid bay scallops were successfully produced following the treatment of fertilized eggs with 6DMAP on April 7th 2003 (97%), April 22nd (88% & 90.7%), May 23rd (80%), June 28th (77%) and July 31st (80%).

Our work revealed that an eleven-minute, 400 µM 6DMAP treatment is sufficient to induce a high percentage of triploidy. To get this high a percentage using the safer reagent, 6DMAP, is practically unheard of in the literature. Ploidy analysis of 90 day-old scallops from the fifth spawn by Rutgers University showed a percentage triploidy of 97% and 100%.

Blake Barton a Masters student from the North Atlantic Fisheries College, Shetland, UK assisted with the spawning and culture of the scallops obtained after the second and the third spawn. During his stay, he monitored the comparative growth between diploid and triploid larvae as his master's thesis project.

Figure 8 shows the comparative survival between triploid and diploid larvae from spawning to setting for our last spawn of the season. This graph is representative of most survival patterns displayed by the populations produced for this experiment. The triploids show very high mortality at first due to the treatment but there no major difference in survival after the first drain down.

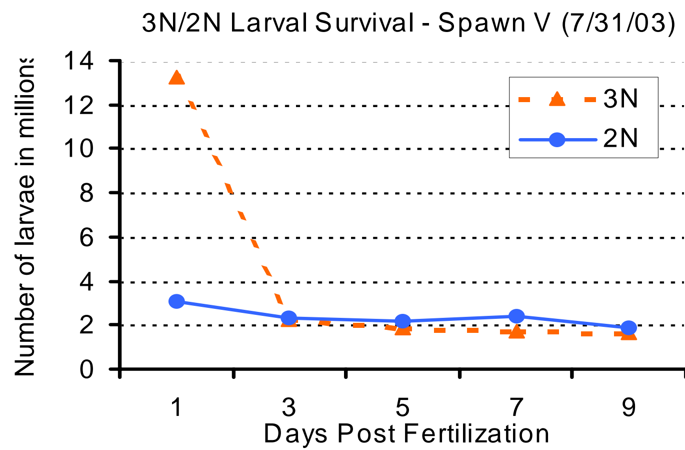


Figure 8. Larval survival of triploids versus diploids during early development for spawn V

Larval and Early Juvenile Culture

Phytoplankton food cultures were grown in sterile F/2 media. The larvae were grown in aerated conicals until metamorphosis and then set in downwellers in a closed, recirculating system. Metamorphosis occurred in 1-2 weeks. Upon setting the larvae were placed on 115 micron sieves with a slow flow of recirculated water supplemented with cultured phytoplankton food. Post-set scallops were eventually moved to downweller sieves with a flow of filtered ambient seawater.

Figure 9. Phytoplankton jugs

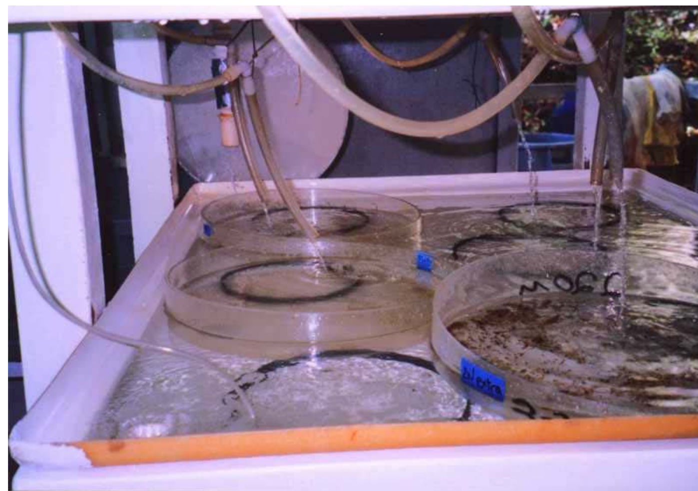


Figure 10. Downweller flow through system.

Figure 11 and 12. The post set scallops were grown on sieves and sorted by size every other day.

The triploid and diploid control juveniles from the first three spawns experienced extremely high mortalities when about 30 days old. They did not survive passed day 40.

The larvae resulting from the fourth and fifth spawnings on June 28th and July 31st 2003 were cultured without problems and resulted in the production of approximately 220,500 triploid and 218,500 diploid (control) seed scallops.

The first two cultures died in heated recirculated systems, possibly due to Vibrio bacterial infection. The third culture died in an open flow through system, using a methodology that has successfully produced millions of seed scallops annually at the MVSG hatchery. The cause for the mortality is being investigated and may be due to unusual dinoflagellate and/or bacterial blooms associated with the extremely wet and cold spring weather.

In July, the water quality seemed to improve and became more favorable for scallop culture. The juveniles from the 6/28/03 spawn survived better than the previous spawns. On August 8th, they were separated into two size groups (2mm and 4mm) and transferred to Jack Blake's tidal upweller. The scallops remained in the upweller until they were deployed in the different experimental sites.

Seed scallops from the July 31st 03 spawn (V) were cultured in raceways at the MVSG Shellfish Hatchery. As we had enough animals from the fourth spawning (IV) for the growth trials, these surplus scallops were distributed to researchers interested in triploidy. These included Rob Garrison of the Wampanoag Aquinnah Shellfish Hatchery (134,500 2N and 3N scallop seed), Ximing Guo from Rutgers (140,000), Dale Leavitt from Roger Williams University (RWU) (30,000), Jim Widman of the National Marine Fisheries Service (45,000). Also, MVSG is attempting to overwinter 9,000 3N and 9,000 2N scallops in pearl nets at the hatchery site.

Approximately 23,000 healthy triploid scallops and 23,000 diploid controls were deployed in growth trial experiments at four sites on Martha's Vineyard, at Moonstone Oysters in Rhode Island, Cornell Cooperative Extension on Long Island, NY, and at the Massachusetts Maritime Academy in Buzzards Bay.

Picture 13 and 14. The juveniles from the 6/28/03 spawn were separated into two size groups (2mm and 4mm) and transferred to Jack Blake's tidal upweller.

<u>Field Culture Trials</u> - On August 26th and 27th 2003, four groups of 3,000 triploid scallops from spawn IV were deployed, with their 3,000 diploid control, on four sites on Martha's Vineyard: in bottom cages at two different densities and hanging bags.

On September 3rd a group of 4,000 3N and 6,000 2N scallops from the same spawn was sent via FEDEX to Cornell Cooperative Extension in Long Island, NY. At this site, they were deployed in pearl nets at three different densities (50/bag, 100/bag, 200/bag).

On September 4th 2003 another group was sent via fast ferry to Moonstone Oysters, in Point Judith Pond, Narragansett, Rhode Island. The scallops were subdivided into four equal subsets per ploidy and placed in polyethylene mesh bags. The bags were placed in two cages suspended one foot below a floating raft in about 5' of water.

On September 3rd 2003 the last group of 3,000 scallops from the June 28th spawn was sent to Massachusetts Maritime Academy's teaching hatchery. The scallops were subdivided into two equal subsets per ploidy and placed in large upweller bins.

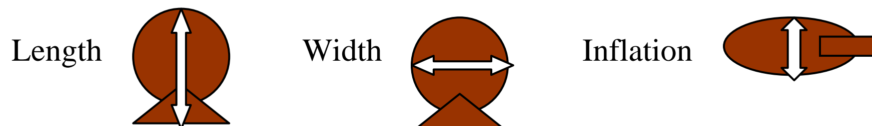
Surplus scallops from the June 28th spawn were distributed to Dale Leavitt of RWU and Scarlett Blair, a private grower on Martha's Vineyard. The scallops did not survive at those sites.

Figures 15 and 16. Triploid and control groups bagged and ready to be send via FEDEX to Moonstone Oysters; Jack Blake deploying a bottom cage at the Katama Bay site, MA.

Figures 17 and 18. Robert Rheault deploying the triploid and control scallops in suspended cages.

Monitor Growth and Survival

From the deployment date, two replicates of 25 scallops per ploidy were measured every two or three weeks depending on the site. Three measurements were recorded for each scallop (length, width, inflation) on specific data sheets created for that purpose.



The measurements were recorded with electronic calipers on site along with survival, temperature and salinity every other week until mid-November. The data sheets were sent to MVSG in a timely manner from the other experimental sites. The data collected was analyzed. Daily growth was calculated and compared between diploids and triploids at each site.

Picture 19, 20, 21 and 22. Measurements were recorded at all sites with electronic calipers during the fall 2003.

Growth and survival data was successfully collected from the grow-out sites and transferred onto electronic spreadsheets for statistical analysis.

First Year Results (2003):

Figure 23 shows the experimental scallops shell growth (Length (mm)/day) for four of our sites: Katama bay, MA, Cedar beach, NY, Buzzards bay, MA and Point Judith, RI.

The results of the analysis of variance showed that there was no significant difference in early growth between ploidy at any of the sites. They also showed that growth was significantly greater at Jack Blake's site in Katama Bay than any other site.

A late ploidy analysis showed that triploidy was lower than expected in spawn VI and had dropped from 77% on July 23rd to 55% on November 17th.

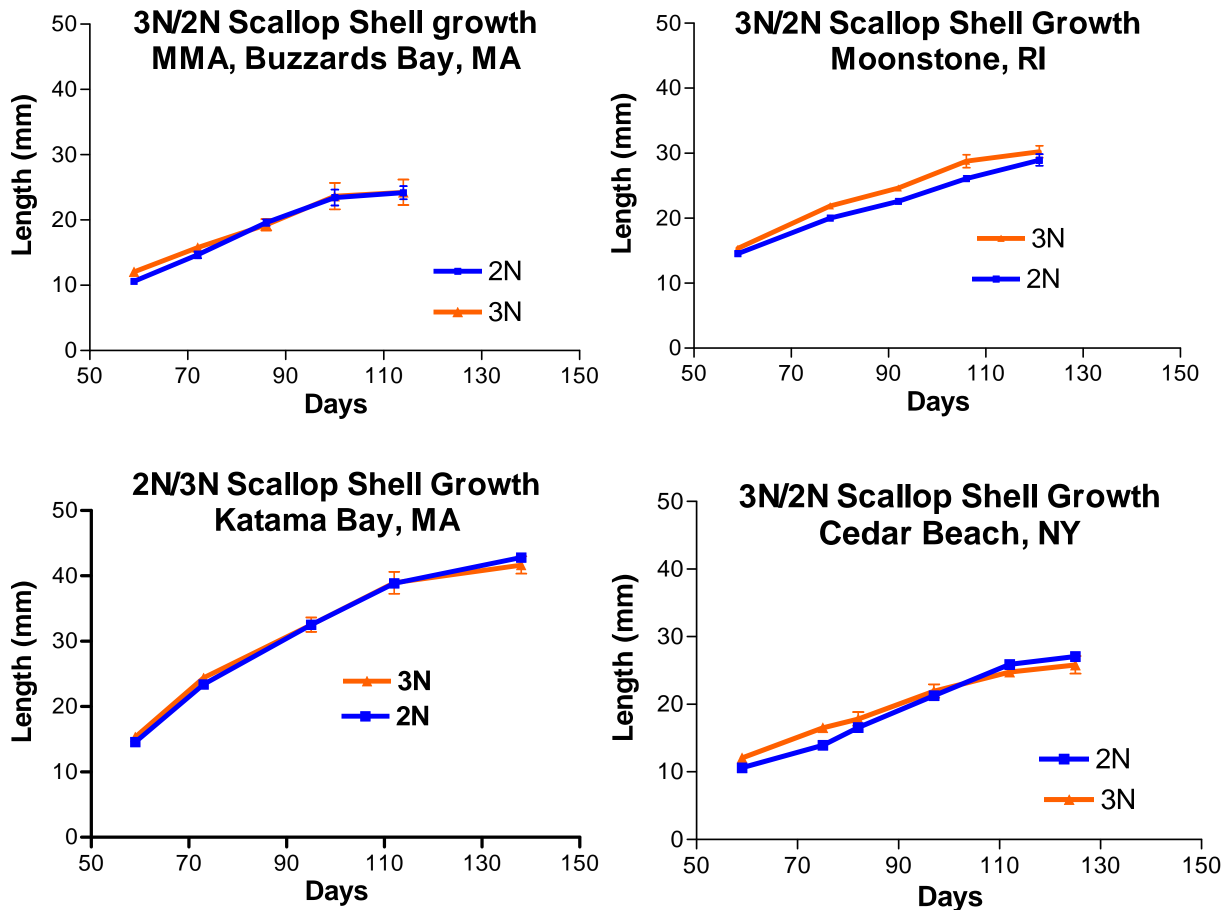


Figure 23. Triploid versus diploid scallops shell growth (Length(mm)/day) 2003 for Katama bay, MA, Cedar beach, NY, Buzzards bay, MA and Point Judith, RI.

As the ploidy level in the experimental scallops from spawn IV dropped down to 55%, the results from the first year monitoring were inconclusive.

<u>Over-wintering:</u>

The scallops from spawn IV were over-wintered at all sites but were not included in the second year growth trials due to their low percentage of triploidy. The scallops from spawn V were stocked in pearl nets for over-wintering and hung on long lines in Lagoon pond at the MVSG site (100 per tier) and in menemsha Pond at the WASH shellfish farm (200 per tier). The animals from spawn V survived well at WASH and MVSG. A late ploidy analysis showed that these animals were still 100% triploid.

The winter was particularly hard in New England and the over-wintering sites in Menemsha Pond and Lagoon Pond were iced over. In March, as the ice started to melt and shift, all but 5 strings of nets holding the triploid scallops in Menemsha Pond were lost and in Lagoon Pond the ice dragged the entire line toward shore and into the mud. Surprisingly, the survival in the strings that remained at the Menemsha Pond site was almost 100 % in the

top tiers. The mortality seemed higher in some of the bottom tiers (possibly due to the strings touching the bottom). At the MVSG site, the survival was very poor, only around 5%, possibly due to the ice, a lack of flow while the animals were in the mud, heavier biofouling on the nets, or the fact that the scallops were smaller when they were deployed for over-wintering.

Figure 24 and 25. Scallops were overwintered in pearl nets in Menemsha pond during the winter 03/04.

Enough 9 month seed scallops from spawn V survived overwintering to be deployed at all experimental sites for the second year growth monitoring (2004)

<u>Spring Deployment and Summer Monitoring (04):</u>

At the end of April and the beginning of May of year II (2004), triploid scallops from Spawn V, the high triploidy spawn, and their diploid control were deployed at each site: two bottom cage sites in Katama Bay on Martha's Vineyard; the WASH site in Menemsha Pond where they were grown both in pearl net and in bottom cages; a site at Moonstone Oysters in Rhode Island (Point Judith) and one at Cornell Cooperative Extension on Long Island. NY (Cedar Beach). Another 3,000 triploid scallops and their diploid control were deployed at the MVSG site in Lagoon Pond.

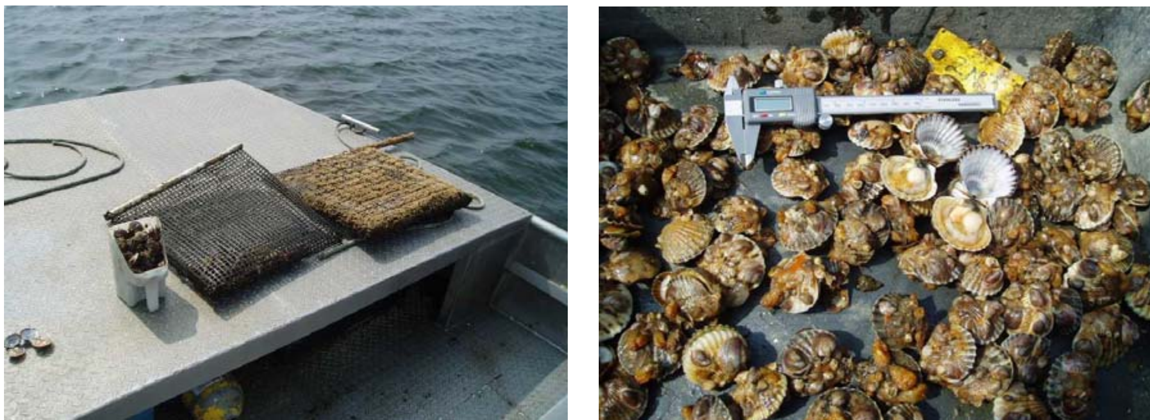


Figure 26 and 27. Deployment and monitoring of one year old scallops at Point Judith (04).

Second Year Scallop Monitoring (2004):

The scallops deployed were monitored from April to November 04.

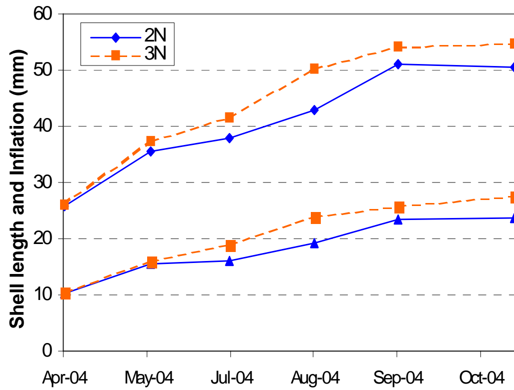
Shell Growth

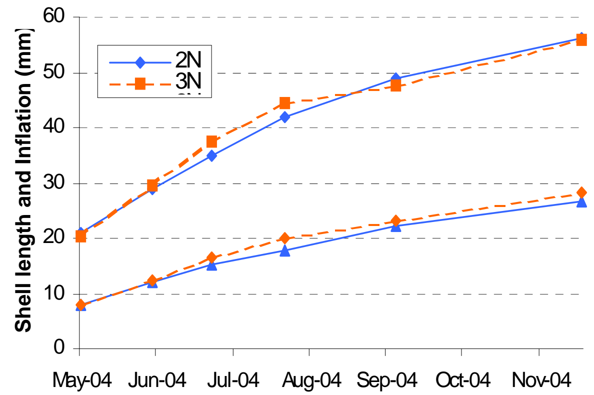
Shell size (shell length and inflation) was recorded every month at each site except the Menemsha Pond site where the hydraulic winch broke making it impossible to pull the experimental cages until the fall. The data was recorded on special worksheets distributed to all participants. Two measurements were recorded: shell length and inflation defined as represented on diagram below.

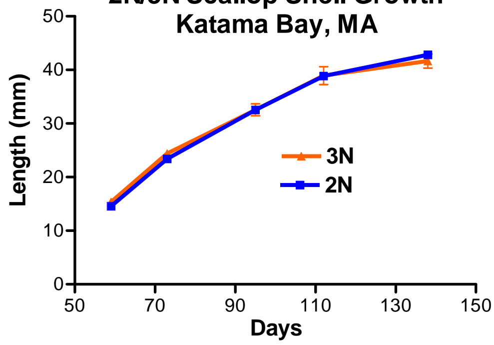
The graphs below, Figure 28, show the experimental scallops' shell growth)Length and Inflation (mm/day) for four of our sites: Katama Bay and Lagoon Pond at MVSG site in MA, Cedar Beach, NY and Point Judith, RI.

The triploids' growth curve consistently stays above the diploids'growth curve at all sites.

The analysis of variance at all sites and dates shows that the triploid scallops' shell size is consistently larger than the diploid control although the significance of the difference varies with date and site. Maximum difference was found during the summer months ranging from 16.8% larger shell length in Cedar Beach NY, to 6% in Lagoon pond MVSG site (9% in Katama bay and 9.7% in RI). Inflation seems to follow the same pattern with an even larger T-values and smaller P values.







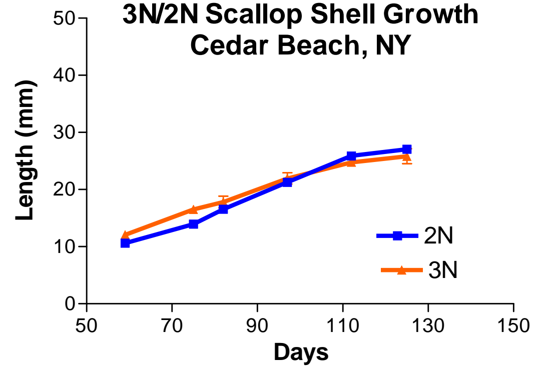


Figure 28. Evolution of shell size of triploid and diploid bay scallops for the growing season 2004 at Point Judith, RI, Lagoon pond MVSG, MA, Cedar Beach, NY and Katama Bay, MA.

Table 1. Summary of the 2 sample t-tests comparing mean scallop shell length and inflation of diploids and triploids in Katama bay by time of sampling.

Sampling date			Length (mm)			
2004	**2N**	**SE Mean**	**3N**	**SE Mean**	**T- value**	**P**
4-28	**27.30**	**0.70**	**28.77**	**0.73**	**-1.45**	**0.156**
6-3	**33.32**	**0.53**	**35.13**	**0.65**	**-2.14**	**0.036**
7-6	**40.78**	**0.65**	**44.76**	**0.70**	**-4.16**	**0.000**
8-11	**46.96**	**0.57 9%**	**51.21**	**0.59**	**-5.18**	**0.000**
9-22	**53.86**	**0.53**	**56.23**	**0.41**	**-3.47**	**0.001**
10-11	**56.49**	**0.79**	**59.69**	**0.62**	**-3.15**	**0.002**
			Inflation (mm)			
	2N	**SE Mean**	**3N**	**SE Mean**	**T- value**	**P**
4-28	**10.58**	**0.30**	**11.44**	**0.33**	**-1.94**	**0.06**
6-3	**13.61**	**0.25**	**14.54**	**0.28**	**-2.45**	**0.017**
7-6	**17.48**	**0.29**	**19.74**	**0.31**	**-5.36**	**0.000**
8-11	**21.18**	**0.30**	**23.5**	**0.25**	**-6.01**	**0.000**
9-22	**24.67**	**0.3**	**27.46**	**0.31**	**-6.51**	**0.000**
10-11	**26.38**	**0.35**	**29.17**	**0.30**	**-5.94**	**0.000**

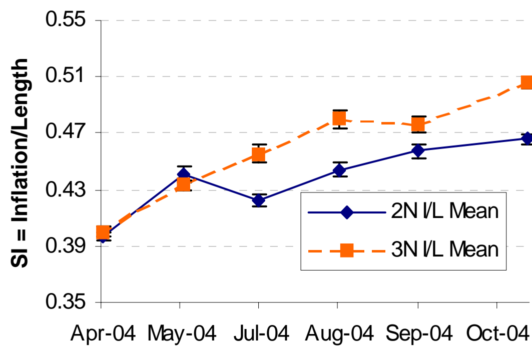
Shell Shape – Spherical Index

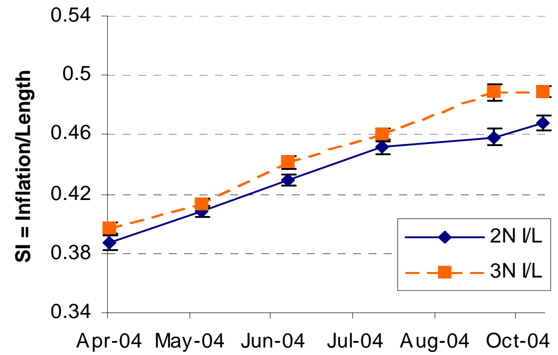
It was shown above that Length and Inflation of triploids tend to be larger than the diploid controls. It also seems that the difference is more pronounced for the Inflation than for the Length measurement.

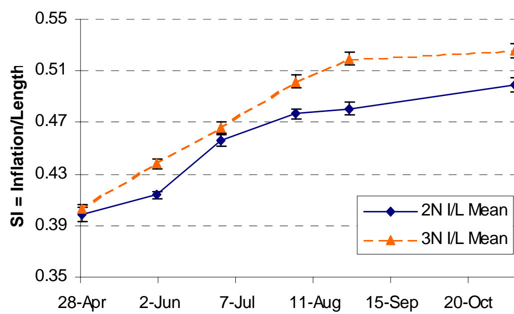
Could this mean that not only the size but also the shape of the shell differs in triploid bay scallops? To analyze this difference, shell shape was defined by the Spherical Index: SI = Inflation/Length. A large SI is associated with a rounder scallop whereas a small SI is associated with a flatter scallop.

Maximum difference was found in the fall, ranging from 6.5% in Katama Bay, MA to 8.5% rounder in Cedar Beach NY.

The results, Figure 29 and Table 2, show that triploid and diploid groups grew rounder as they grew larger. SI was significantly greater in the triploid groups in the late summer/fall which means that 3N scallops were significantly rounder than 2N controls.







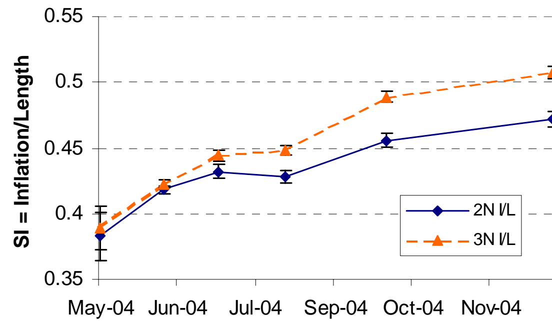


Figure 29. Evolution of shell shape of triploid and diploid bay scallops defined by the Spherical Index SI = Inflation/Length for the growing season 2004 at Point Judith, RI, Lagoon pond MVSG, MA, Cedar Beach, NY and Katama bay, MA.

Table 2. Summary of the 2 sample t-tests comparing mean scallop spherical index of diploids and triploids in Katama bay by date of sampling.

Date	2N(I/L) Mean	SE Mean	3N (I/L Mean	SE Mean	T-Value	P
29-Apr	0.3876	0.0048	0.3973	0.0037	-1.62	0.114
3-June	0.4082	0.0034	0.4139	0.0031	-1.25	0.216
6-Jul	0.4292	0.0041	0.4418	0.0043	-2.12	0.038
11-Aug	0.4512	0.004	0.4606	0.0039	-1.7	0.094
22-Sep	0.4586	0.0051	0.4886	0.0052	-4.11	0
11-Oct	0.468	0.005	0.4891	0.0041	-3.24	0.002

Gonad and Adductor Muscle Weight

In late June and July, during the period of sexual maturity, samples were taken from four sites on Martha's Vineyard and dissected in the laboratory. Three body components were separated (adductor muscle, gonad and viscera), dried and weighed separately to establish a wet and a dry adductor muscle and gonad weights and indexes.

In late October and November, during the commercial harvesting period, all sites were sampled and adductor muscle and gonad weights and indexes calculated again.

Figure 30 and 31.Three body components were separated (adductor muscle, gonad and viscera), dried and weighed separately.

Wet versus Dry weight

The comparison of dry and wet weight values gives an idea of the water content present in adductor muscle and gonad of triploid and diploid scallops in the summer and fall months. Table 3 and Figure 32 shows that the water content for the adductor muscle is around 75% on average and varies very little from summer to fall/winter. Water content is 6 to 16% higher in the gonad than in the adductor muscle. Unlike the adductor muscle that does not change with the season, the gonadal tissues contain 10% more water in the fall/winter than in the summer. These results confirm visual observations of "watery/spent" gonad after the end of the summer season. There does not seem to be a difference in water content between diploid and triploid bay scallops.

Table 3. Percent dry weight versus wet weight of triploid and diploid bay scallops adductor muscle and gonad. (Values = (dry weight / wet weight) * 100)

% dry weight								
	Adductor Muscle				**Gonad**			
	Summer		**Fall**		**Summer**		**Fall**	
	2N	3N	2N	3N	2N	3N	2N	3N
MVSG	23%	24%	22%	25%	20%	19%	10%	8%
Aquinnah	24%	24%	-	-	21%	17%	-	-
Jack	23%	26%	27%	28%	20%	16%	11%	10%
MVSG II	23%	24%	-	-	18%	14%	-	-
Scott	25%	25%	-	-	21%	16%	-	-
Cornell Coop.	-	-	17%	20%	-	-	9%	9%
Moonstone	-	-	24%	25%	-	-	10%	9%
Mean % dry weight	23%	24%	22%	24%	20%	17%	10%	9%
Mean Water content	**77%**	**76%**	**73%**	**76%**	**80%**	**83%**	**90%**	**91%**

Wet and dry adductor muscle and gonad weight

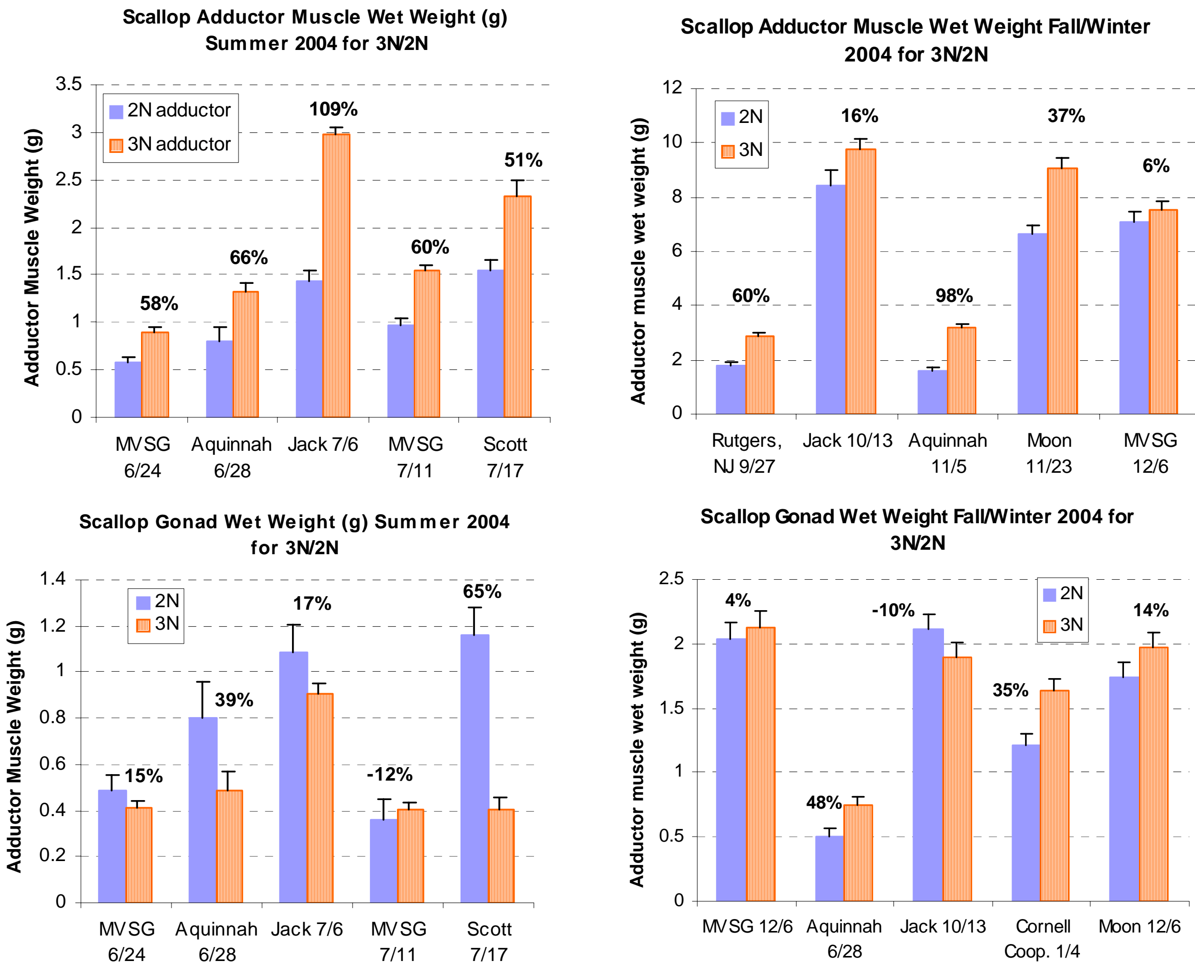


Figure 32. Comparison between 3N and 2N wet adductor muscle and gonad weight for the summer and fall samplings of the 2004 growing season.

Table 4. Summary of the % difference in weight of 3N versus 2N 1-year-old bay scallops for the summer and fall of the 2004 growing season.

% difference in wet weight of 3N versus 2N								
	Adductor Muscle				**Gonad**			
	Summer		**Fall**		**Summer**		**Fall**	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
MVSG	58% *	60% *	6%	17%	-15%	-16%	4%	-11%
Aquinnah	66% *	72% *	-	-	-39% *	-50% *	-	-
Jack (Katama)	109% *	135% *	16% *	20% *	-17% *	-33% *	-10%	-20% *
MVSG II	60% *	66% *	-	-	12%	-10%	-	-
Scott (Katama)	51% *	51% *	-	-	-65% *	-73% *	-	-
Cornell Coop.	-	-	56% *	87% *	-	-	35% *	26% *
Moonstone	-	-	37% *	42% *	-	-	14%	6%
Rutgers	-	-	60% *	-	-	-	-	-

The value are in 3N % difference from 2N (+ value = 3N larger, - value = 3N smaller). The values marked with a * are significant ($P<0.05$).

Adductor muscle wet weight in triploid scallops was significantly larger than that of diploids at all sites and dates except the MVSG fall sampling. The difference ranged from 6% to 109% larger. The difference between 3N and 2N adductor muscle weight was even more significant once the meat was dried. The difference ranged from 17% to 135% larger for dry weight.

Figure 33. Diploid scallop (left) shows larger gonad, smaller muscle and smaller overall weight the triploid scallops (right).

Triploid gonad wet weight was significantly smaller than that of diploids at 3 out of 5 sites at the summer sampling. The other 2 sites, MVSG I and II did not show any significant difference in gonad weight.

In the fall, results are a little more unexpected. Only the 3N gonad dry weight at Jack Blake’s site in Katama proves to be significantly smaller that that of diploids (-20% dry weight) whereas the New York site at Cornell Cooperative extension (Cedar beach) shows a significantly larger gonad weight for triploids (26% dry weight). This surprising result might be due to the much larger overall size of triploids versus diploids at that site tipping the balance towards a larger gonad although the proportional size of the gonad is smaller.

The adductor muscle and gonad index values brings more light on the proportional weight of each components in 3N versus 2N.

Wet and dry adductor muscle and gonad indexes

Wet and dry Adductor muscle indexes where calculated according to the following equations:
Adductor muscle index = Adductor muscle weight (g) / total body weight (g)
Gonad Index = Gonad weight (g) / total body weight (g)

Adductor Muscle Index:

The study of the adductor muscle and gonad indexes allow to have a better understanding of the relative effects of triploidy on the different parts of the scallop, especially the effect on the commercially important adductor muscle.

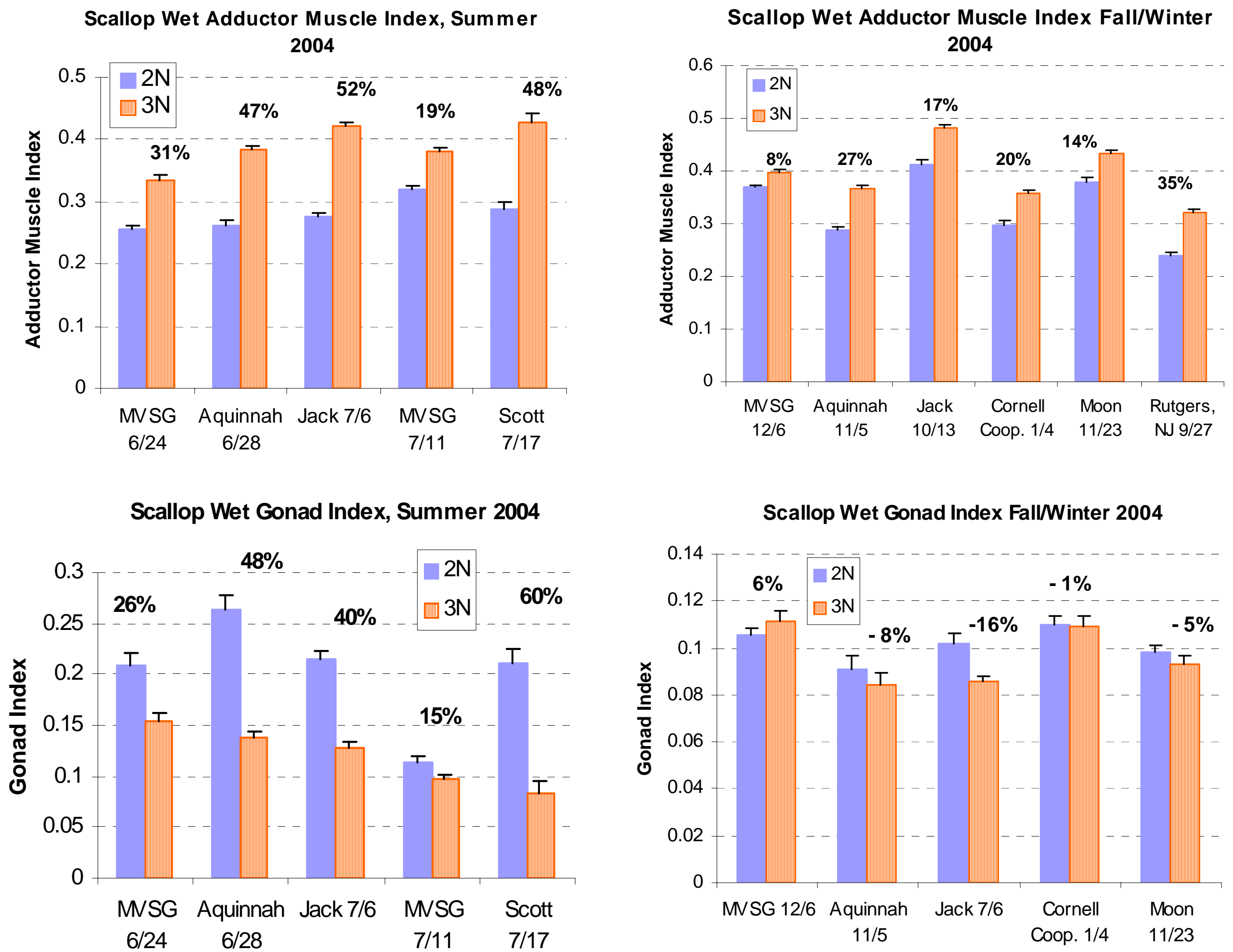


Figure 34. Comparison between 3N and 2N wet adductor muscle and gonad indexes for the summer and fall samplings of the 2004 growing season.

Percentage difference in index of 3N versus 2N was calculated according to the equation:
% difference in index of 3N versus 2N = (3N index – 2N index) / 2N index

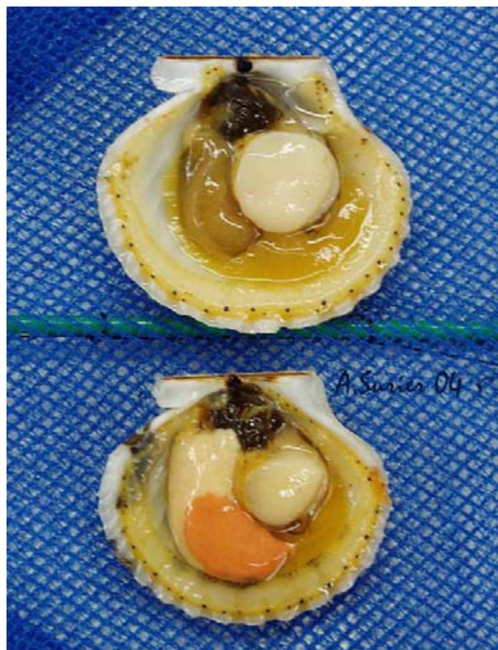
The results show that triploids adductor muscle indexes were significantly greater than diploid controls at all sites regardless of the sampling season. At all sites the results were highly significant. Wet adductor muscle indexes ranged from 19% to 52% larger in the summer and 8% to 27% larger in the fall/winter. Dry indexes showed similar results.

Triploidy had the opposite effect on gonad indexes which were significantly smaller than those of diploids at all sites during the summer months ranging from 15% to 60% smaller. At all sites the results were highly significant. In the fall/winter, after the spawning season, the differences between triploids and diploids gonad indexes where less pronounced with still four out of five sites showing statistical significance when comparing dry indexes.

Table 5. Percent difference in index of 3N versus 2N 1 year old bay scallops for the summer and fall of the 2004 growing season.

% difference in index of 3N versus 2N								
	Adductor Muscle Index				**Gonad Index**			
	Summer		**Fall**		**Summer**		**Fall**	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
MVSG	31%*	28%*	8%*	5%*	-26%*	-30%*	6%	-28%*
Aquinnah	47%*	44%*	27%*	33%*	-48%*	-55%*	-8%	-11%
Jack	52%*	54%*	17%*	9%*	-40%*	-56%*	-16%*	-29%*
MVSG II	19%*	20%*	-	-	-15%*	-28%*	-	-
Scott	48%*	51%*	-	-	-60%*	-68%*	-	-
Cornell Coop.	-	-	20%*	23%*	-	-	-1%	-14%*
Moonstone	-	-	14%*	12%*	-	-	-5%	-14%*
Rutgers	-	-	35%*	-	-	-	-	-

The value are in 3N % difference in Index from 2N (+ value = 3N larger, - value = 3N smaller). The values marked with a * are significant ($P<0.05$).



Picture 35. Triploid scallops (top) grew larger and had larger adductor muscle index than diploid controls (bottom).

These results mean that triploidy does not only increase the overall size of scallops but also it significantly increases the proportion of the commercially interesting part of the scallop while reducing the proportion of the waste product.

Triploid Gonad Development Observations

Unexpected findings

During the experiment, the triploid scallops were not expected to ripen due to the impossibility for cell to achieve proper meiosis when holding an uneven number of chromosomes. However, some of the gonads of the triploid group showed some signs of development.

To determine if triploid scallops can undergo gonad maturation and what the frequency of such an event is, 50 scallops from the triploid population were sampled on July 12th and 15th. The state of gonad development was scored by visual inspection using gross morphological criteria: immature, partially ripe, ripe and spent. Out of 50 scallops, 7 showed signs of gonad development. Each scallop was then bled and the hemolymph samples collected were sent to the Virginia Institute of Marine Science for flow cytometry analysis to make sure that all scallops observed were indeed triploid. Out of the 50 scallops analyzed, one was a diploid.

The 7 ripe triploid scallops where kept on a flow through system until the next spawn. They were added to the broodstock during the spawning event. They did not spawn with the rest of the broodstock.

Although the majority of triploids failed to ripen during the summer months, 12% showed fully or partially developed gonads but did not spawn when stimulated by heat shock.

Chemical Analysis of Adductor Muscles

On December 7th, 25 triploid and 25 diploid scallops were sampled from the Martha's Vineyard Shellfish Group site. The animals were shucked and the adductor muscles were sent to the Brooklyn University laboratories for glycogen, protein and carbohydrate analysis.

Material and Methods

Twenty-five samples of diploid and triploid bay scallop adductor muscles were shipped on ice from Martha's Vineyard Shellfish Group on 8 December 2004 and received at Brooklyn College, The City University of New York (Brooklyn, NY), on 9 December 2004. Each adductor muscle was weighed (Mettler-Toledo AE 163) to determine the wet tissue weight (±0.0001g). The samples were then placed into a drying oven (Thelco 26) at 60°C until a constant weight (±0.0001g) was reached to determine dry weight. The individually dried tissue samples were ground to a fine powder using a mortar and pestle. Portions of this ground tissue from individual scallops were then used to determine average carbohydrate, protein and lipid levels.

The total content of carbohydrates (glycogen is the most prominent carbohydrate stored in marine bivalves) in each adductor muscle was determined (in triplicate) using the phenol – sulfuric acid method of Dubois et al. (1956). A 2.0-5.0 mg portion from each

sample was placed in a test tube with 10 ml of distilled water. The solution was then homogenized. Three 1.0 ml samples were taken from this solution and placed into three separate test tubes. Distilled water was added to each to a final volume of 2.0 ml. A 50 µl aliquot of 80% phenol reagent was added to each tube and vortexed. A 5 ml aliquot of 95% sulfuric acid was then rapidly added to each tube. The samples were allowed to stand at room temperature for 30 minutes. The absorbance of all samples was read at 490 nm on a Hach DR/2010 spectrophotometer. A calibration curve was constructed using glucose as a standard.

A modification of the Bradford (1976) method (commercial kit, Pierce Biotechnology, Rockford, IL) was used to determine protein content in triplicate. A portion of dried tissue (4.0 –8.0 mg) from each sample was placed in a test tube with 10 ml of 0.1N NaOH, homogenized, and left overnight at room temperature. Three 50 µl aliquots from this solution were placed into three separate test tubes and 1.5 ml of Coomassie Plus™ protein assay reagent was added to each tube and vortexed. The tubes were then incubated for ten minutes at room temperature. The absorbance of the samples was read at 595 nm on a Hach DR/2010 spectrophotometer. A calibration curve was constructed using bovine serum albumin as a standard.

Total lipid content was estimated, with two to three replicate samples, using the method of Folch et al. (1957). A portion of dried tissue (0.05-0.23 g) was placed in a test tube with 5 ml of 2:1 (v/v) chloroform-methanol mixture and was left to stand for one hour. The solution was then made biphasic by adding 1 ml of distilled water (20% of chloroform-methanol) and mixed. The mixture was centrifuged at 3000 rpm to further separate the two phases. The upper phase was typically 40% and the lower phase 60% of the total volume of the system. A 2 ml sample of the lower phase (chloroform with lipids) was placed on a previously combusted and weighed aluminum weigh boat. The weigh boat was then placed on a clean surface in the fume hood with a glass plate above the weigh boat to prevent dust contamination. After the chloroform had evaporated, the weigh boat was placed into an oven at 110°C for 30 minutes. The weigh boat was then placed in a dessicator until cool and weighed. Total lipids were calculated by subtracting the weight of the weigh boat from the weight of the weigh boat plus lipids and then multiplying by the proportion of chloroform in the original system and divide that product by the weight of tissue used in the sample. A blank was created by mixing 5 ml of 2:1 (v/v) chloroform-methanol and 1 ml of distilled water and then measuring the volume of chloroform (lower layer), which yields the proportion of chloroform in the original system.

All results were expressed as the mean percentage (± standard deviation) of biochemical component per unit dry weight. Mean biochemical content of the diploid and triploid groups were compared using a two-tailed t test of difference between means (Zar, 1999). The level of significance was set at $P < 0.05$. All data collected are shown in Appendix 1-6.

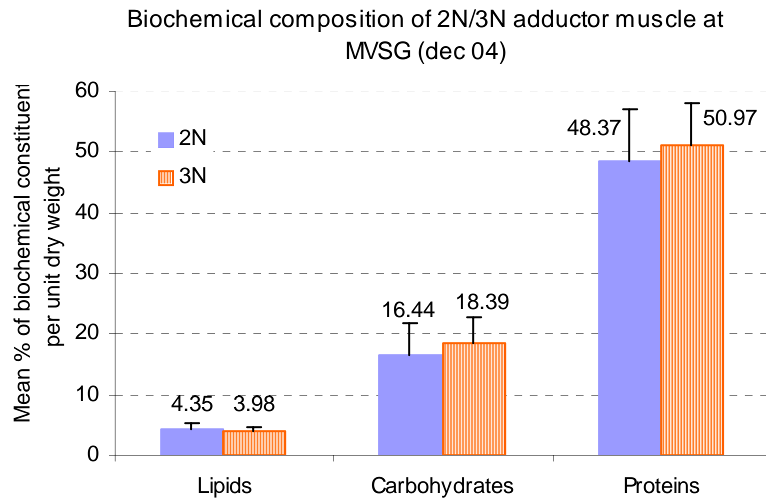


Figure 36. Biochemical composition of triploid and diploid adductor muscle in dec. 04.

Results

Analysis of the total protein, carbohydrate and lipid content of triploid and diploid bay scallop adductor muscle yielded no statistically significant difference between the means of the two strains (Fig. 1). The mean protein content of adductor muscle in the triploid bay scallops sampled was 50.97% (± 7.0). The adductor muscle of the diploid bay scallops sampled had a mean protein content of 48.37% (±8.56). The triploid strain had a mean carbohydrate content of 18.39% (±4.20), which was slightly higher, although statistically insignificant, than the diploid strain that had a mean carbohydrate content of 16.45% (±5.23). The total lipid content in the diploid strain was found to be 4.35% (±0.85). The triploid strain had a slightly lower lipid content of 3.98% (±0.54).

Analysis of the total protein, carbohydrate and lipid content of triploid and diploid bay scallop adductor muscle showed no statistically significant difference between triploids and diploids.

The results obtained from these analyses in terms of total carbohydrate, protein and lipid content of the adductor muscle in *Argopecten irradians irradians* were similar to the results described in other studies of *Argopecten irradians irradians* (Epp et al. 1988), *Argopecten irradians concentricus* (Barber & Blake 1981) and *Argopecten ventricosus* (Ruiz-Verdago et al. 2001; Palacios et al. 2004).

The results demonstrated the similarities between the diploid and triploid strains in terms of protein and lipid content. This has also been observed by Palacios et al. (2004) in diploid and triploid strains of *A. ventricosus*. However, Palacios et al. (2004) observed a significant difference in total carbohydrate content between triploid and diploid *A. ventricosus,* with the triploid having a greater proportion than the diploids. The authors suggested that this was due to a lack of transference of carbohydrates from the muscle to the gonads in the sterile triploid scallops. In addition, Tabarini (1984) found a significantly greater in triploid *Argopecten irradians* than in diploids at the end of the growing season in Maine, USA.

The discrepancy between the results obtained in this study and the aforementioned studies may be explained by the timing of the sampling.

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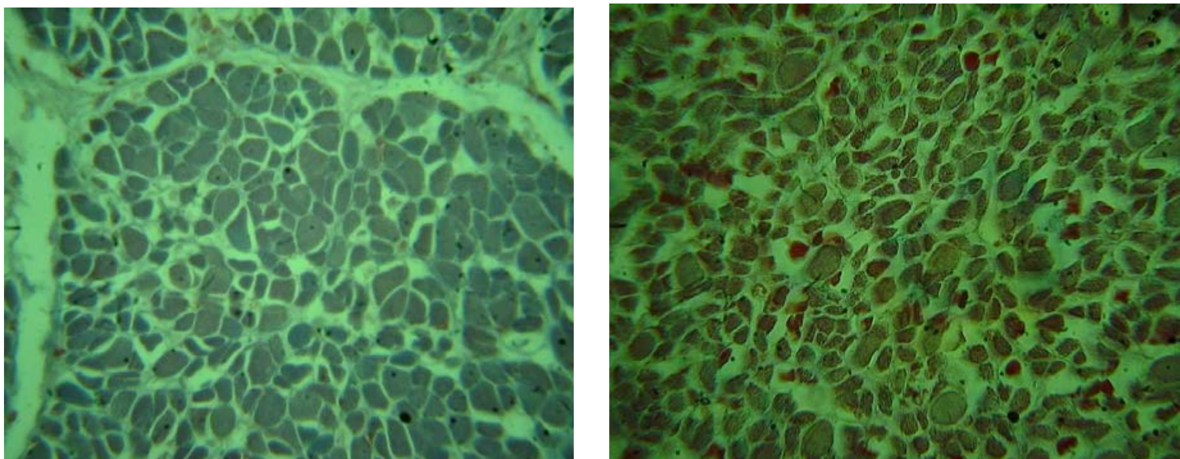
Cell properties analysis

Objective

To compare the size of adductor muscle cells in diploid and triploid bay scallops, *Argopecten irradians*, cultured at Martha's Vineyard Shellfish Group (Oak Bluffs, MA).

Material and Methods

A portion of the adductor muscle was fixed in Bouin's solution, embedded in Polyfin™, sectioned transeversely (7μm), and stained with Masson trichrome stain (Presnell and Schreibman, 1997). The slides were observed and photographed using a microscope (x40) and the diameter of individual striated muscle fibers (Chantler, 1983) was analyzed using NIH ImageJ software. To ensure precision of measurements three different images representing a different section were analyzed by measuring the diameter of the first thirty muscle fibers observed in both the triploid and diploid adductor muscles. In addition, a criterion was established to limit subjectivity, in which muscle fibers characterized as round and granulated were selected for measurement. All other muscle fibers (i.e. oval and/or non-granulated) were not measured.



Picture 37. Transversal section of adductor muscle of triploid (left) and diploid (right) bay scallops.

Table 6. Striated muscle fiber cell diameter in Triploid and Diploid bay scallops.

Bay Scallop muscle fiber cell diameter

Sample #	Mean size (μm)	SD	n
2N1	5.45	±1.13	30.0
2N2	6.48	±1.33	30.0
2N3	5.91	±1.17	30.0
2N4	6.37	±1.32	30.0
2N6	6.95	±1.65	30.0
2N7	7.49	±1.63	30.0
2N8	7.07	±0.98	30.0
2N10	5.62	±1.27	30.0
2N11	5.34	±1.00	30.0
2N Mean	6.30	±1.28	270.0

Sample #	Mean size (μm)	SD	n
3N1	7.6	±1.1	30.0
3N2	8.7	±1.7	30.0
3N3	6.0	±1.1	30.0
3N4	8.6	±1.8	30.0
3N5	7.8	±1.8	30.0
3N6	6.79	±1.2	30.0
3N Mean	7.6	±1.5	180.0

A two sample t-test showed that triploid scallop muscle fibers were significantly larger ($p = 0.00$), averaging 7.58 μm ± 1.75 whereas the diploid control muscle fibers measured 6.30 μm ± 1.47 on average.

Table 7. Muscle fiber counts per unit area in Triploid and Diploid bay scallops.

Bay Scallop cell count per unit area

Sample #	Mean count	SD	n grids/fields	Sample #	Mean count	SD	n grids/fields
2N1	67.89	±5.51	9	3N1	±64.67	6.56	9
2N2	73.11	±17.14	9	3N2	±40.89	6.29	9
2N3	70.67	±5.36	9	3N3	±73.22	5.93	9
2N4	52.33	±6.28	9	3N4	±53.33	14.20	9
2N6	70.67	±4.00	9	3N5	±52.11	7.75	9
2N7	36.78	±6.26	9	3N6	±55.67	5.92	6
2N8	55.44	±7.26	9	3N Mean	±56.65	7.78	
2N10	73.44	±8.11	9				
2N11	67.89	±11.66	9				
2N Mean	63.14	±7.95					

In contrast, the difference in number of muscle fibers per unit area was not significant ($p = 0.45$).

Both set of results suggest that triploid muscle fibers are larger and more tightly packed than diploid muscle fibers.

Outreach

2004

The project progress and early results were presented at the NMFS Aquaculture Seminar in Milford, Connecticut and at the World Aquaculture Society (WAS) meeting in Honolulu in the spring of 2004.

The abstract from the Milford presentation was published in the June 04 edition of the Journal of Shellfish Research.

A magazine article entitled "Improving Shellfish Aquaculture through Triploidy" written by PI Amandine Surier and published in the June 04 edition of the Global Aquaculture Advocate mentions the NRAC scallop triploidy trials.

The project progress and preliminary results were presented in a poster at the Northeast Aquaculture Conference and Exposition 2004 (NACE 04) in Manchester, New Hampshire.

2005

Final results were presented through oral communications at Aquaculture America (Jan 18th 05) in New Orleans, Louisiana, the NMFS Aquaculture Seminar (Feb 05) in Milford in Connecticut and the National Shellfisheries Association meeting (April 05) in Philadelphia, PA.

2006

Further results were presented as a poster at the National Shellfisheries Association Conference

in Monterey, CA. (see poster below)

A publication is being currently written for the Journal of Shellfish Research.

A first draft of the industry outreach publication has been written and reviewed by the co-author.

Progress Reports

Abbreviated Progress Report

Project Code: 03-15 **Subcontract/Account No: 556806**

PROJECT TITLE: Developing and Testing Novel Methodology for Land- and Near Shore-Based Aquaculture of the Green Sea Urchin.

FUNDING LEVEL: Year 1: January1, 2004-December 31, 2004 **- $68,321**
Year 2: January 1, 2005-December 31,2005 - **$46,891**
Year 3: January 1, 2006-December 31, 2006 - **$54,438**

PARTICIPANTS (FUNDED TO DATE):

Dr. Walker – PI, project coordinator, coordinator of laboratory components, evaluating gonadal histology;
Mr. Devin – CoPI/ Consultant/Service Provider- land and near shore husbandry and technology transfer;
Dr. Böttger – Post-Doctoral Student - Production of triploid sea urchins;
Mr. Tamaki - President and Founder, I.S.F. Trading Co. Inc. - Taste testing of green se urchins.

PROJECT OBJECTIVES:

Objective I. To use invariant photoperiod to produce green sea urchins for the winter and summer Japanese markets with gonads that do not initiated gametogenesis, contain only nutritive phagocytes and are of high commercial quality (Years 1-2).
Objective II. To develop sterile triploid green sea urchins for use in either commercial land- or near shore-based aquaculture that contain only nutritive phagocytes and are of high commercial quality (Years 1-3).

Progress and Principle Accomplishments:

Objective I: We have conducted a study related to objective I and aimed at producing green sea urchins for the winter Japanese market. We used one trough system (holding approximately 500 animals) and five cages (each containing 100 sea urchins) maintaining urchins respectively in invariant (July, using specially designed lighting systems) photoperiod in the lab and at ambient photoperiods at the lease site of the Darling Marine Center, Maine. During the six months (July-December, 2004) of this experiment, urchins were fed an extruded diet prepared for this purpose by Wenger Mfg. Inc. at the end of the experiment, gonad indices had increased significantly in both treatments, from 4.79% initially to 23.47% for ambient and 20.5% for invariant photoperiod treatments. Following these results we had taste testings of ten animals from each treatment conducted by Mr. Atsuchi Tamaki of I.S.F. Trading Company Inc., who evaluated urchins maintained at ambient photoperiod as having usable roe of 17% (compared to total body weight), consistent color/ texture and sweet taste. animals maintained at invariant photoperiod, however, were evaluated as having usable roe of 10%, inconsistent color/texture and a bitter taste. The taste results have to lead to the following considerations regarding sea urchin aquaculture. First of all, the index that we commonly use for scientific experiments with sea urchin gonads (wet weight gonad/wet weight whole animal *100) does not necessarily reflect the final value of roe sold on the market. During the scientific evaluations all small amounts of gonad will be extracted, however not

so in the sea urchins harvesting process, which is reflected in the amount of usable roe, as determined through Mr. Tamaki. Second, the extruded Wenger diet will yield gonads of large size compared to preliminary experiments, where animals fed a diet of *laminaria saccharina* for five months and yielded gonad indices of 16.67% under ambient and 15.02% under invariant light regime. However, these gonads were not considered to be of good taste and color and will thus not yield a high market price (discrepancies between animals maintained under ambient and invariant photoperiod are a result of sea urchins feeding on encrusting organisms as well as the extruded diet in the ambient photoperiod treatment).

Results for stereology of gonads (**Fig. 1**) showed a significant increase in the volume fraction of nutritive phagocytes under invariant photoperiod from initial values (37.92% in males and 10.36% in females) to those in urchins at the end of the study (80.34% in males and 73.8% in females). There was no change in the volume fractions of nutritive phagocytes in urchins maintained under ambient photoperiod. Sizes of nutritive phagocytes increased in males and females from both invariant and ambient photoperiod treatments. When expressed as the nutrient portion of the nutritive phagocytes (excluding developing gametes in both sexes), we again found an increase in males and females under invariant and ambient photoperiod. However, the nutrient portion of NPs in individuals under invariant photoperiod were significantly higher than those of individuals under ambient photoperiod. Volume fractions of gonial cells increased from 20.12%$\pm$ 5.51 in males and 0% in females initially to 37.81% $\pm$ 1.78 in males and 22.56%$\pm$ 1.04 in females in individuals maintained for five months under ambient photoperiod.

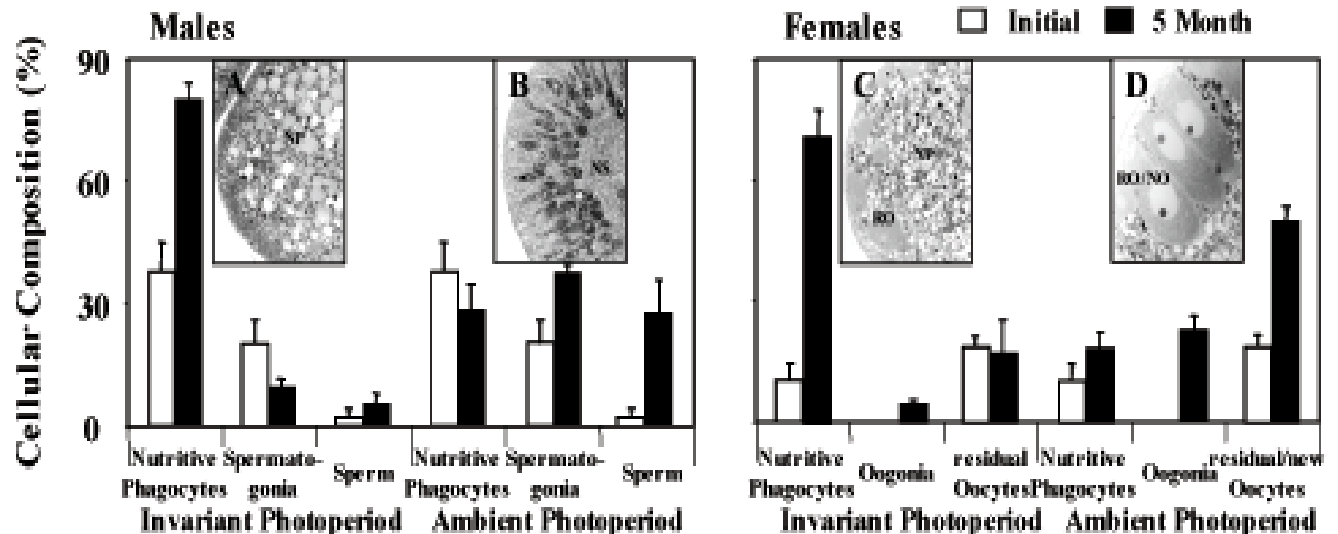


Figure 1 - Volume fractions (%) of the NPs, gonial cells, oocytes and sperm of green sea urchins evaluated for males and females at time zero and after culture for five months under initial and ambient photoperiod. Animals were sacrificed in July (initial) and after 5 months (December). Pictures (A) and (B) for males and (C) and (D) for females represent invariant and ambient photoperiod respectively. NP = nutritive phagocyte, NS = new sperm, RO = residual oocyte, RO/NO = residual and new oocytes.

Under invariant photoperiod, there was a decrease in the volume fraction of gonial cells to 9.74% $\pm$ 2.11 in males and no change in females. Volume fractions of luminal spermatozoa and of primary oocytes within NP incubation chambers also increased in individuals maintained under ambient photoperiod from 2.15% $\pm$ 1.34 to 28.04% $\pm$ 7.85 in males and from 18.62% $\pm$ 2.61 to 49.67% $\pm$ 3.64 in females. No significant change in volume fractions of spermatozoa or residual oocytes in individuals maintained under invariant photoperiod was recorded. Size frequencies (%) of oocyte diameters increased significantly in urchins maintained under both invariant and ambient photoperiod. Under invariant photoperiod the mean oocyte diameter of residual oocytes from last years gametogenesis increased to 93.51 µm $\pm$ 3.66 (from 56.21 µm $\pm$.

2.23 initially) Under ambient photoperiod, mean oocyte diameters increased to 125.96 µm ± 7.34. These results indicate that we can manipulate the cellular composition of sea urchin gonads using invariant photoperiod to a composition that is favored by the Japanese consumers. However, the commercial diets currently available produce roe of an unpalatable taste, which lead us to test new experimental diets (produced through cold extrusion) formulated and manufactured at Texas A&M by Addison Lawrence and Steve Watts.

For this experiment we tested seven new diets and compared them to the currently available commercial diet (used previously) in the aquaculture module, feeding 50 urchins on each diet for 5 months. After five months there we noted an increase in gonad indices with all diets, including the commercially available diet (**Fig. 2**). Three of the new diets were scored as marketable by our taste tester, their color evaluated as very good, and while taste scores are still not as high as from naturally harvested urchins, we are making progress in identifying a diet that will produce roe of high market value.

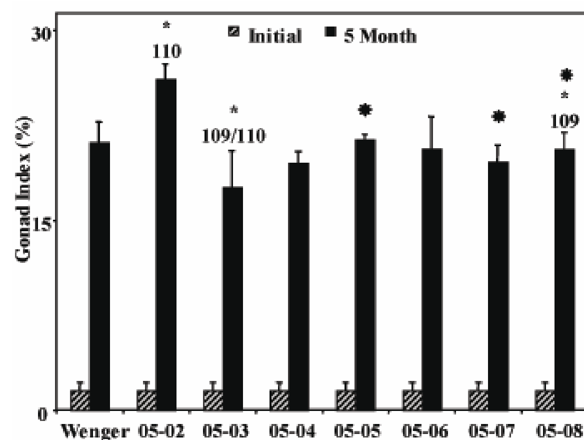


Figure 2 - Gonad indices (% wet weight) for green sea urchins fed eight different feeds (commercially available Wenger and experimental feeds formulated and prepared by UAB and Texas A&M). * = consistent color, # = orange/yellow color on the Maine DMR chart (laboratory dissection), ✷ = deemed marketable by the taste tester (Atchan Tamaki, ISF Trading).

Objective II: We have successfully removed the jelly coat and vitelline membranes and achieved maximum fusion rates for 2 fully mature ova. Removal of the vitelline membrane was accomplished by spawning female sea urchins (injection of 2 ml 0.5 M KCl) an incubating the eggs for 30 minutes in calcium-free seawater with 0.2% cysteine and 1.2 mg/ml pronase. Denuded ova were then treated with either polyethylene glycol and poly(Arg). Polyethylene glycol did not yield fusion. Ova treated by dilution to 75% of their original volume with dH_2O plus $CaCl_2$ to a final concentration of 25mM and followed by a 10-fold with a solution of 0.0075 mg/ml poly(Arg) in calcium-free seawater consistently yielded >40% fusion of eggs in 1.5 ml microcentrifuge tubes following 50 minute exposure. However, when fertilization of these fused ova was attempted no development was accomplished, leading us to revise our treatments of the ova.

We have therefore modified the procedure for the removal of the jelly coat and vitelline membrane using a mechanical and an acidic method. The mechanical method entails an egg suspension of 1:10 eggs in calcium free seawater (which will weaken the jelly coat) that is filtered through a 210µm nylon mesh 9-10 times. The mesh size had to be slightly larger than the eggs (164.21µm ± 6.12) to ensure that the eggs were not damaged in the process. For the acidic methodology eggs were incubated for 50 minutes in filtered seawater at pH 5. Of the three methods we have now employed for the removal of the jelly coat (including the chemical removal with pronase that seemed to affect subsequent fertilization), the chemical treatment is

still the most successful (98.1% $\pm$ 1.9 SE), however, since it seems deleterious to fertilization we have chosen the acidic removal, which had a success rate of 79.7% $\pm$ 6.2 removal over the less reliable mechanical removal (47.8% $\pm$ 17.8).

We then followed fertilization and development through to gastrula stage, when we measured the size of normal (fertilized without removal of vitelline membrane and fusion) and potential triploid embryos (that had undergone acidic removal of the jelly coat and successful fusion). Results showed that normal embryos were significantly smaller 197.5 µm $\pm$ 5.71, than embryos that had undergone fusion (234. 5 µm $\pm$ 2.34). Triploid embryos were consistently obtained from these fusions based on their size (normal embryos were significantly smaller 197.5 m $\pm$ 5.71 than embryos that underwent fusion 234.5 m $\pm$ 2.34) and chromosomal number (**Fig. 3**). These embryos were raised to normal blastula stage only (Böttger et al., in preparation).

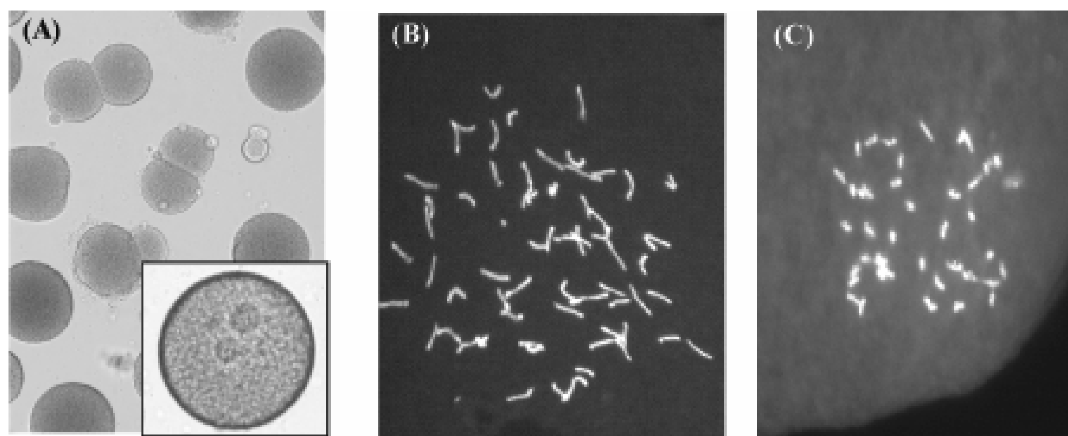


Figure 3 - Production of triploid eggs and blastulae. (A) Eggs undergoing fusion, inset fused eggs with two pro-nuclei prior to fertilization; (B) 63 chromosomes in a triploid blastula (fused egg fertilized by normal sperm) at 24 hrs after fertilization; (C) 42 chromosomes in a normal blastula 24 hrs after fertilization.

WORK PLANNED: At this point we are planning to disseminate our work, through publications, presentations and updating the website for Dr. Charles Walker.

Impacts: The development of techniques for successful large-scale production of urchins with suppressed gametogenesis (following either objective I or II) will be a considerable boon to sea urchin aquaculture in the following ways. Large-scale land-based culture of sea urchins depends upon understanding and manipulating gametogenesis to yield gonads of maximum size, texture and taste. The use of invariant photoperiod to produce such gonads is simple and can be employed in small or large-scale aquaculture ventures at a variety of sites on the East Coast. Production of triploid sea urchins would permit similar results in near-shore lease sites where photoperiod cannot be controlled.

SUPPORT:

Year	NRAC-USDA Funding	University	Industry	Other Fees	Other Total	Total Support
2004	$ 68,321	$ 30,542	0	0	0	$ 98,863
2005	$ 46,891	$ 10,970	0	0	0	$ 57,788
2006	$ 43,618	$ 10,820	0	0	0	$ 54,438
Total	$158,830	$ 51,549	0	0	0	$210,289

PUBLICATIONS, MANUSCRIPTS OR PAPERS PRESENTED:

Publications

Novel methodology for generating triploid green sea urchins – Applications for open-ocean aquaculture. S. Anne Böttger, Celeste C. Eno and Charles W. Walker. In preparation for Aquaculture.

Suspension of gametogenesis in green sea urchins experiencing invariant photoperiod – Applications for Aquaculture. S. Anne Böttger, Michael G. Devin and Charles W. Walker. Submitted to Aquaculture.

Böttger S.A., C.W. Walker and T. Unuma. 2004. Care and maintenance of adult echinoderms. In: Methods in Cell Biology, Volume 74, Development of sea urchins, ascidians and other invertebrate deuterostomes: Experimental approaches. Academic Press. pg.17-38.

Presentations

Two novel culture approaches for sea urchin aquaculture. To be presented at the Annual NACE, Mystic, CO, December 2006.

Novel methodology for generating triploid green sea urchins – Applications for open-ocean aquaculture. To be presented at the 12th International Echinoderm Conference, Durham, NH, August 2006

Application of photoperiod manipulation and new extruded diets in aquaculture of the green sea urchin (*Strongylocentrotus droebachiensis*). NSA Annual Meeting, Monterey, CA, February 2006

Current echinoderm research: Novel approaches using photoperiod manipulation and triploidy for culture of the green sea urchin. GOMMEA Annual Meeting, Portland, ME, November 2005.

Application of novel technologies in sea urchin aquaculture: Photoperiod manipulation and triploid production. Research Seminar, June 15, 2005. The University of Maine at Orono, ME.

Culture of the green sea urchin: Novel approaches using photoperiod manipulation and triploidy. Research Seminar, March 2, 2005. Roger Williams University, RI.

Assessment of two novel culture methods for land- and near shore based aquaculture of the green sea urchin (*Strongylocentrotus droebachiensis).* Annual NACE, Concord, NH, December 2004.

Normal and altered gametogenesis in the green sea urchin Strongylocentrotus droebachiensis: Implications for aquaculture. Sea urchin 2003, International conference on fisheries and aquaculture, March 24-29, 2003, Puerto Varas, Chile.

PROGRESS REPORT

Project Code: 04-4 **Subcontract/Account No:** 557222

Project Title: Nutrition Studies in Hatchery Technology of Clownfish and Pygmy Angelfish Culture in Closed Marine Systems.

Reporting Period: 15 July 2006

Funding Level: $81,042.00 Year One; $86,339 Year Two

Participants:

Principal Investigator(s) and Brief Statement of Qualifications:
Harold Pomeroy, Professor of Biology, Roger Williams University, 10 years experience in finfish aquaculture.
Bradford D. Bourque, Marine Laboratory Manager, Roger Williams University,
Masters degree in aquaculture, 7 years experience with marine finfish and shellfish culture
Andrew M. Lazur, Associate Professor/Extension Specialist, University of Maryland Center for Environmental Sciences Horn Point Laboratory, Over 20 years experience in finfish aquaculture research
Bill Van Huekelem, Associate Research Scientist, University of Maryland Center for Environmental Sciences Horn Point Laboratory, Over 20 years experience in finfish and shellfish aquaculture research
Joe Soares, Owner, Black Duck Farm Ltd., producer of marine ornamental species
Retired (emeritus status) professor in fish nutrition, University of Maryland

Project Objectives:

1) Evaluate effect of broodstock nutrition on reproduction and egg quality
2) Evaluate the effect of copepod nauplii as a supplemental diet during the period of first feeding
3) Determine the effect of juvenile diet on coloration, growth and survival
4) Compile experimental results into an optimized production protocol for the species and use them to conduct an economic feasibility analysis
5) Dissemination of information learned from experiments to industry and public by tours, publications, presentations and workshops

Anticipated Benefits: The global trade of marine ornamental species for the aquarium industry is valued at over $7 billion annually. In the United States, ornamental fish are the second largest pet industry with over $660 million worth of live freshwater and marine fish imported in 1998. There are estimated to be 13 million aquaria in U.S. households, of which 1.1 million hold marine ornamentals. This constitutes an annual trade valued at $300 million that includes over 25 million individual specimens. Of these, 95% are still obtained from the wild. Culturing aquarium species in closed systems minimizes environmental impact and leads to the production of hardier specimens that survive longer in captivity.

Aquaculture of these species has the potential to supply a superior product that is in high demand while relieving pressure on threatened populations. The largest domestic market for marine ornamentals is the Northeastern U.S. (Ewart 1997). This high value, close proximity to markets, ability to easily make suitable saltwater with commercial formulations thereby providing applicability of recirculating systems in any location, makes marine ornamental fish an obvious choice for the northeast region. Fewer than half a dozen commercial companies currently produce significant quantities of marine ornamental specimens for the retail trade, and none of these are located in the Northeast. One of the largest costs to producers is shipping these specimens over long distances. Up to 25% of the shipment can die during prolonged transport, which reduces the profit margin considerably. Close proximity to the major market is a significant advantage. In the case for Florida's freshwater ornamental industry, several hundred small family operations produce numerous species and sell either to large producers/distributors or direct to consumers. The same scenario is possible with marine ornamental in the northeast.

Marine ornamentals have the potential to be the high value alternative aquaculture species needed to create a viable aquaculture industry in the northeast. However, a bottleneck for expansion into marine ornamental fish culture is the limited number of species for which the technology of captive reproduction has been fully developed. Of the 1475 species currently traded, only about 30 have been successfully reared commercially. Nutrition is a key bottleneck in aquaculture production and development. Without proper nutrition, spawning, larval survival, and juvenile growth are compromised. Broodstock diet is a critical component of producing high quality eggs, which will have the energy reserves that larvae require to begin feeding. Also essential is having a first feed that larvae will consume that meets the nutritional requirements for optimal growth. Once the fish reach the juvenile stage a producer needs the fastest growth possible while also achieving desired coloration and morphology. It is expected that the results of the various objectives will significantly add to the understanding of improving broodstock and larval nutrition requirements, a major bottleneck currently experienced with culture of marine ornamental fish. In addition, the ability to see production system requirements and the economic analysis will provide valuable guidance into other investment options for small producers in the northeast region, potentially reducing risk. The outreach from this study will target farmers and be a practical guide including system designs, species considerations, spawning and larval rearing technology and recommended management practices.

Progress and Principal Accomplishments:

Objective 2.1A: Broodstock diet impact on egg production, hatch rate, and larval survival

Horn Point Lab:
There has been considerable success with the brood stock diet study involving two diets; MadMac-MS maturation simulator diet and Gelly Belly. Horn Point experienced the same decline in clownfish brood stock spawning during the early summer, however pairs are now producing. They are analyzing nest size, percent hatch and survival of larvae produced by fish fed the two diets. Analyses should be complete in the fall of 2006.

Roger Williams:
The second round of brood stock diet experiments, which consists of 3 parts, with 15 pairs of *Amphiprion ocellaris,* was initiated on April 1, 2006. Part 1 ended on July 14, and part 2 was started on August 1 and will end on September 15. Three treatment feeds (Gelly Belly modified Hoff gelatin-based diet, Formula One Gel diet and Tetra Color Tropical Marine Flake) were used with five replicates per treatment. Pairs were weaned onto diets for a period of three weeks before the experiment began. Although spawning showed a marked decline for 6 weeks during the early summer, there have been 61 spawns recorded, with 41 spawns resulting in eggs collected for measurement, chemical analysis for fatty acid content, and hatch/survival analyses. Preliminary analyses has yielded the following: Formula One Diet; spawns per pair 4.5, days to hatch 9.25, hatch rate 6.9%, survival to 30 days 76.8%. Tetra Marine Flake; spawns per pair 5.7, days to hatch 8.6, hatch rate 4.2%, survival to 30 days 24.7%. Gelly Belly Diet; spawns per pair 6.0, days to hatch 9.0, hatch rate 3.3 %, survival to 30 days 62.2%. The third part of the final set of experiments on brood stock diet will start on September 31, 2006 and end on November 15.

Objective 2.1B: Effect of copepod nauplii as a supplemental diet during the period of first feeding

Horn Point Lab:
One more trial of rotifer enrichments will be conducted since the lab had an issue with the quality of enrichments used last year. However, work indicated that copepods are not necessary since survival and growth has been very good with enriched rotifer and Artemia, followed by introduction of flake diets. Horn Points work indicated that an extra cultured food, such as copepods, seems unnecessary.

Roger Williams:
Two sets of experiments have been conducted since January 2006. In the first set of experiments three larval treatment diets were tested: copepod nauplii (*Acartia),* rotifers (*Branchionus*), and a rotifer/copepod mix. Three replicates of 10 fry each were used for each treatment. In all cases newly hatched fry were fed a treatment diet until day 7, then weaned onto newly hatched artemia, to 72 hour enriched artemia on day 9, a 300 micron diet on day 16, and larval flake diet on day 21. The experiments concluded on day 30, with survival and growth recorded. The experiment was run twice, starting on 4/7/06 with fry from brood pair OC-6, and again starting on 6/4/06 with fry from the same brood pair. Preliminary analyses indicate that although growth may have been slightly better with the

copepod diet, survival was better with the mix and best with the rotifer diet. A second series of experiments focused on copepod versus rotifer early diets. Five replicates of 10 fish each were initially fed copepod nauplii as in the first experiment, and five replicates of 10 fish were initially fed rotifers. Each replicate lasted 30 days, at which time survival and size measurements were made. The rotifer treatment had survivals of 52% - 60% with a mean length of 1.37cm, while the copepod nauplii treatments had survivals of 31% - 40%, with a mean length of 1.48cm. Results were similar to the initial study, with slightly greater growth observed with the copepod diet, but survival greater with the rotifer diet. Our findings agree with those noted by the Horn Point experiments in that the extra effort of growing copepods is not essential for clownfish culture.

Objective 2.1C: Effect of pigment supplement diets on coloration, growth and survivability of juvenile clownfish and angelfish

Horn Pont Lab:
As reported in their presentation at Marine Ornamentals '06, the work at Horn Point Lab with juvenile diets as the affect growth and coloration of clownfish indicates the advantage of addition of Cyclop-eeze to a standard TetraMin Troical Flake diet greatly enhances coloration. It should be noted that a diet for optimum color did not produce maximum growth in their experiments, and thus any marketing strategy would require consideration of diet sequence in order to produce the maximum number of both highly colored and fastest growing fish. Horn Point has continued the work on juvenile diet and plans to have a manuscript draft in September 2006 in preparation for submission this fall.

Roger Williams:
We have completed our final experiments on juvenile feeding. Results were reported at Marine Ornamentals '06. We have continued the experiment with the original four treatment diets (Marine Color Flake, Formula One Flake, Total Color Marine Flake and Cyclop-eeze) where 3 of the 6 replicates of 30 fish were continued on the original diet and 3 of the replicates were switched to Cyclop-eeze out until day 240. Analyses are being done on growth (both weight and length) as well as color analysis. The data will be submitted as a manuscript for publication during the fall 2006 academic semester.

Studies involving flame angelfish, Centropyge loriculus

Two 200 gallon recirculating systems, each to be stocked with 2 male and 4 female angelfish have been establish and conditioned at RWU. The first system has held six angelfish for 12 months. We have had success spawns and have collected eggs for hatching in order to conduct first feeding experiments. We have successfully reared larval angelfish to day 8, but have not had survival past this stage. Stomach content analyses are being conducted to establish if the larval fish are eating the copepod nauplii (a mix of several species all of which seem to be 60+ microns). We are currently working with a lab at UCONN which will provide ciliates of 40 micron size so that we can run experiments in which first feeding is centered on ciliates.

Impacts:

The future of marine ornamental aquaculture lies not with species like clownfish which have bee produced for 30 years, but with species not yet cultured. Less than 3% of the species currently traded have been successfully reared commercially. The key bottleneck has bee larviculture. This involves appropriate feed, but also encompasses broodstock nutrition to ensure appropriate egg quality and hatch rates. The impact of these experiments will be twofold:

- To incorporate the numerous developments in systems designs and nutrition which have occurred or the past decades into experiment in the areas of broostock, larval and juvenile culture and nutrition in clownfish, focusing on maximizing output with available technologies and feeds. Our first analyses would indicate that: the time and expense of copepod culture is not necessary for successful aquaculture of clownfish, and also that diets do not vary significantly in terms of survival, but have notable differences on growth and coloration of juvenile clownfish.
- To then adapt some of these findings to development of successful culture protocols for flame angelfish and other commercially important marine ornamental fish species.

Recommended Follow-up Activities:

- Test and adapt protocols developed by these experiments to attempt to culture other species of marine ornamentals.
- Evaluate economic significance of findings of these experiments by quantifying the impact of increased production efficiency. This is especially affected by our findings on larval survival (copepods versus rotifers) and juvenile diet studies. Preliminary indications are that the slight increase in expense of Cyclop-eeze may be offset by both greater number of clownfish "crops" (4 rotations every two years as opposed to 3 rotations on diets resulting in slower growth), and better coloration. Note that our studies will indicate that switching to Cyclop-eeze 30 days before fish are brought to market will result in positive color enhancement, however growth of fish using alternate feeds is significantly slower.
- Evaluate experimental alternatives determined from observation (Eg: new feed types, system designs, culture methodology, disease prevention, induction and control of reproduction, water quality parameter requirements).

Support:

YEAR	NRAC-USDA FUNDING	UNIVER-SITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	TOTAL SUPPORT
1	$ 81,042	$ 32,776					$ 113,818
2	$ 86,339	$ 34,877					$ 121,216
TOTAL	$113,818	$121,216					$ 235,034

Publications, Manuscripts, Or Papers Presented since February 2006:

COMOS; The Society for Small Molecule Science, conference held in Las Vegas – July 2006:

A MORE EFFICIENT METHOD OF FATTY ACID ANALYSIS AND THE DETERMINATION OF CAROTENOID CONTENT IN THE STUDY OF BROOD STOCK AND JUVENILE NUTRITION IN MARINE ORNAMENTAL FISHES

Stephen K. O'Shea*, Nancy E. Breen, Bradford D. Bourque, Amanda White, Kevin Jackson, Loong Fat Ho and Harold F. Pomeroy

The successful breeding and growth of tropical marine ornamental fish has been demonstrated to be very closely related to the diet. Polyunsaturated and highly unsaturated fatty acids (PUFAS and HUFAS) and carotenoids are important components of marine fish diets and are critical for egg and larval development and survival of offspring. The determination of fatty acids has traditionally been conducted using FAMES and GC/MS. This presentation demonstrates a new more sensitive and time efficient method using HPLC/MS technology for the determination of fatty acids. This novel application reduces the number of time-consuming preparative steps and also allows for a higher throughput analysis. Current traditional GC/MS methods involve lipid extraction followed by saponification and esterfication conducted in situ which requires a 90-minute time period per sample. Once a sample is prepared for GC/MS analysis it requires a further one-hour per sample analysis run. In our analysis of fatty acids by HPLC/MS, the sample preparation is reduced to 60 minutes and each chromatographic run is reduced to 6 minutes per sample. This time reduction is accomplished partially by the elimination of the need for preparative esterfication of the sample. The time for each chromatographic run is quite short as we are running our HPLC with an isocratic solvent flow and are able to observe the molecular ion of each fatty acid using electro spray as our MS ionization method. The validity and sensitivity of this new protocol was verified by replicating media enrichment feed studies of artemia and analyzing for their fatty acid content in comparison to the standard GC/MS methodology. This new application for HPLC/MS technology was used in the analysis and the determination of fatty acids in false percula clownfish (*Amphiprion ocellaris*) eggs and diets. Four essential long chain fatty acids were quantified: docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid and docosapentaenoic acid. Diets containing high concentrations of carotenoids have been shown in many marine fish to improve vibrancy of external pigmentation and are invaluable metabolic antioxidants. The variety and concentration of carotenoids present in each of the feeds for the brood pairs, grow out experiments, and eggs were determined from their MTBE extraction followed by HPLC/MS analysis. The determination of fatty acids and carotenoid content in a sample of eggs from a clutch was correlated to broodstock diet fatty acids, carotenoid content, hatch rate, egg size, fecundity, and larval survival to determine if dietary fatty acid and carotenoid content have an effect on egg quality in clownfish. Additionally fatty acids and carotenoid content of varying feeds were correlated to external pigmentation and the successful development and growth of juvenile fish. Flake diets were compared to replicates fed cyclop-eeze that have a known high concentration of the carotenoid astaxanthin.

We have also submitted an abstract for the NACE meeting to be held in Groton, CT December 6-8, 2006. We have been accepted for a one hour technical session at the meetings.

This is your Official Notice that the Program Committee of NACE 2006 has accepted your abstract for:

Abstract Number: 55

Presentation Title:
PRODUCTION TECHNOLOGY AND ECONOMIC FEASIBILITY OF MARINE ORNAMENTAL AQUACULTURE

Presenting Author:
Harold F. Pomeroy

PRODUCTION TECHNOLOGY AND ECONOMIC FEASIBILITY OF MARINE ORNAMENTAL AQUACULTURE

Harold F. Pomeroy*, Bradford D. Bourque, Joseph Evans, Loong Fat Ho, Amanda White, Spencer Gowan, Timothy Arcand, and Kevin Jackson
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Marine ornamental fish trade is valued at over 300 million dollars worldwide. Currently less than 2 % of the species traded are cultured commercially. The intense collection pressure on wild organisms and the damaging techniques used to collect them is detrimental to coral reef habitats.

The marine ornamental aquaculture research project at Roger Williams University was started in 2002 to investigate the potential for commercially producing marine ornamentals in the northeast. The project initially investigated species which had already been successfully produced elsewhere. Clownfish (*Amphiprion ocellaris*), seahorses (*Hippocampus erectus*), and peppermint shrimp (*Lysmata wurdemanni*) were produced and evaluated for production efficiency and cost of production. A preliminary market analysis was also conducted.

Experiments were conducted to identify more efficient clownfish production methods in each of the production stages: broodstock, larviculture, and growout. Diets were evaluated for each of these stages for their effect on hatch rate, survival, and growth and coloration, respectively.

The production techniques developed were applied and adapted to additional species. These include fire shrimp, orchid dottybacks, yellowhead jawfish, and flame angelfish.

An economic analysis was conducted which investigates the feasibility of commercial marine ornamental aquaculture. Production cost, efficiency, and market estimates were all entered into a model which used linear programming to determine what volumes of each species to produce. Risk and sensitivity analyses were also conducted. The potential impact of additional species in production was also investigated.

ANNUAL PROGRESS REPORT

Project Code: 05-02

Subcontract/Account No. 557221
supported by 2003-38500-13505

Project Title: Development of genetic markers to assess disease resistance in the eastern oyster

Reporting Period: February 2005 - July 2006

Funding Level: $128,486

Participants:

Steven Roberts – research scientist in MBL's Scientific Aquaculture program with background in molecular biology

Roxanna Smolowitz - traditionally trained veterinary pathologist, has extensive experience studying bivalve disease

Richard Karney – Director of Martha's Vineyard Shellfish Group, Inc., holds culture experience with numerous bivalves

Inke Sunila – invertebrate pathologist, has extensive experience in the diagnosis of bivalve diseases and working with aquaculturists

Dale Leavitt – extensive experience in research and aquaculture of shellfish, hatchery management, and extension

Bill Walton – aquaculture specialist with research experience in shellfish biology and outreach/extension activities

Frederick Goetz – senior scientist with background in aquatic animal immunology and molecular biology.

Project Objectives:

1. To demonstrate seed originating from local wild oysters, that have experienced heavy disease (Dermo) pressure, could significantly contribute to the development of disease resistance in cultured oysters.
2. To genetically characterize regional oysters (C. virginica) that are putatively resistant (more tolerant) to Dermo, in order to development genetic markers and to better understand mechanism involved in immunity.
3. To communicate with northeastern hatchery operations and help them to identify local, potentially Dermo resistant broodstocks.

Anticipated Benefits :

1. Information related to the effectiveness of using of local broodstock (that have experienced heavy disease pressure) to successfully contribute to the regional oyster aquaculture effort.
2. Genetic characterization of C. virginica from the northeast region, that will contribute significantly to our understanding of mechanisms involved in disease resistance and aid in any breeding programs.
3. Simple laboratory tests to identify broodstock that have traits associated with disease resistance.
4. Website, fact sheets, and presentations containing information on how hatcheries can select premium broodstock locally, based on prior disease pressure and genetic markers.

Progress and Principal Accomplishments

One of the major causes of decreased production for the oyster industry is disease. The two primary diseases that affect the eastern oyster are MSX and Dermo. Both diseases invade the oyster's soft body resulting in death of the individual. The disease MSX is caused by the protozoan parasite Haplosporidium nelsoni and is present along the entire east coast. This parasite was originally given the name Multinucleated Sphere with unknown affinity (X). The disease Dermo is caused by the parasite Perkinsus marinus. In the last few years, the disease has markedly affected oyster culture in the more northern portion of the parasite's range (Connecticut, Rhode Island and Massachusetts) in addition to states already identified as problematic (New York to the Gulf of Mexico). Oyster disease is a particular concern of shellfish farmers in the northeast region not only due to periodic devastating oyster losses, but also because disease indirectly affects the industry by slowing financial investments. Realizing that oyster disease is a primary concern for the industry, the long-term goal of the proposed research is to assist in the development of disease resistant eastern oyster broodstocks.

A primary objective of our research is to demonstrate seed originating from local wild oysters, that have experienced heavy disease (Dermo) pressure, could significantly contribute to the development of disease resistance in cultured oysters. Previous research has demonstrated that genetic factors can be selected for that contributes to disease resistance in the eastern oyster. A majority of this work has involved hatchery-based selection practices with limited information on the performance of wild oyster populations that have survived heavy disease pressure.

Therefore our focus is to characterize disease tolerance in local, naturally selected for oysters in relation to factors such as P. marinus infection, growth and genetics.

Figure 1. Temperature data recorded in Edgartown Great Pond at grow-out site.

Oyster broodstock populations were identified on Martha's Vineyard, MA that had experienced heavy disease pressure, and that had not been exposed to Dermo in the recent past. Edgartown Great Pond (ETGP) was selected as the site to obtain oysters that had experienced heavy disease pressure. Edgartown Great Pond is a salt water pond that is separated from the ocean by a sand bar most of the year. As a result, little recruitment occurs from coastal waters.

Dermo prevalence and associated mortality have been very high in the pond for the past eight years, yet old animals are not hard to find. The control, or potentially susceptible oyster broodstock population was obtained from Tisbury, MA. There was limited Dermo occurrence at this site prior to selecting the broodstock. These two broodstock populations were spawned in July 2005, and seed from both broodstock populations was deployed. In July 2005, approximately 8000 oyster seed (4000 from ETGP stock, 4000 from Tisbury stock) were deployed in floating ADPI bags at two sites in Edgartown Great Pond. The average shell length of the oyster seed was 21 mm. In September 2005 the oysters were thinned out by adding additional ADPI bags at each site and evenly distributing the oysters. Since September 2005 there have been 12 bags at each site (6 with ETGP oysters

and 6 with Tisbury oysters). In October 2005, oysters were counted and sixty oysters from each of the two groups were examined for P. marinus occurrence and abundance. P. marinus was observed in approximately twenty percent of all the oysters examined.

However, the average abundance of the parasite in infected oysters was higher in the Tisbury group. **These results could suggest that P. marinus proliferates more in oysters from a population that had not experienced heavy disease pressure.** Floats were removed from the ADPI bags and they were submerged until May 2006 in order to avoid harsh conditions on the surface. In May, oysters were counted and samples brought back to the Marine Biological Laboratory for measuring and Dermo testing.

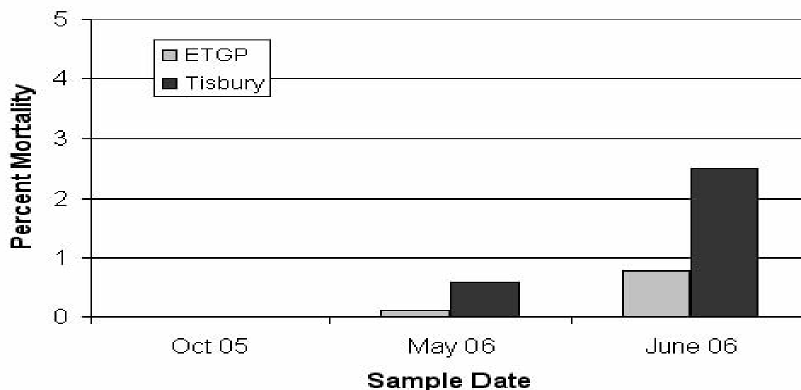

Figure 2. Mortality data for two oyster populations (Edgartown Great Pond and Tisbury) deployed in ADPI bags at our field site.

API bags and they were submerged until May 06 in order to avoid harsh conditions on the surface. In May, oysters were counted and samples brought back to the marine Biological lab measuring and Dermo testing. During the first year of grow-out, survival rates for all oyster groups were greater than 95% (Figure 2). However, increased survival was consistently observed for the Edgartown seed as opposed to the Tisbury group. **Once again, this demonstrates a difference in the EGTP and Tisbury oyster populations that could be associated with differential disease resistance and/or susceptibility.**

Growth was not significantly different between the two oyster groups. As expected, slowest growth rates were observed during winter months with an average overall increase in shell height of 2.8 mm between October and May.

The second objective of this research project is genetically characterize regional oysters that are putatively resistant to Dermo, in order to development genetic markers and to better understand mechanism(s) involved in immune function. To begin to address this issue, we examined gene expression patterns in oysters provided to us by Marta Gomez-Chiarri and Dale Leavitt (participant on current project). Two wild populations were obtained from Rhode Island. These included oysters from Blue Bill Cove (Portsmouth) and Green Hill Pond (Charlestown). The Green Hill Pond strain had survived heavy Dermo pressure, where as the Blue Bill Cove strain had been exposed to very little Dermo pressure, if any at all. A third, proven disease resistant line of oysters were also characterized from the Haskin Shellfish Research Laboratory, Rutgers University. Oysters were kept for approximately one month in the same holding tank and were fed twice weekly. When transferred to a holding tank, several oysters were infected with Dermo.

This led to circulation of Dermo throughout the holding tank and equal exposure to the parasite for all oyster groups. After one month of growth, gill and mantle tissue samples were taken from each oyster for later RT-PCR analysis.

Select genes were identified based on their presumed function in the immune system to characterize in these oysters. Gill tissue was used for RNA extraction due to the prevalence of hemocytes which are the cells primarily responsible for immune function in oysters. One gene that was analyzed using real-time quantitative RT-PCR was a mitogen activated protein (MAP) kinase interacting protein (GenBank Accession number CD526707). The MAP kinase signaling pathway is important in innate immune function in both vertebrates and invertebrates. Interestingly, the level of gene expression was similar for the proven disease resistant strain (Rutgers) and the population from Green Hill Pond that had survived heavy disease pressure in the recent past (Figure 3). **One explanation for this is that there could be similarities in the immune response of the Green Hill Pond oysters and the Rutgers strain.**

The final major component of our work includes outreach. One way we have begun to meet this objective is the development of a website http://www.mbl.edu/aquaculture/oyster). The purpose of this website is to keep the general public and shellfish farmers up to date on our activities and progress to date. This website includes some of our field data and photos of our routine field sampling. There is also a link to a local newspaper article that featured this project. We plan to keep this site up to date with future additions to include a copy of our progress report and presentation given at regional and national meetings.

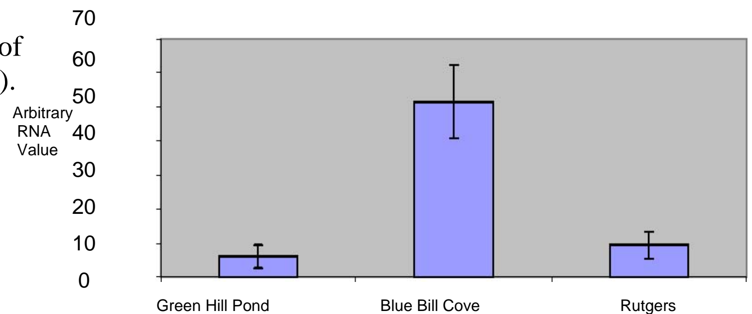

Figure 3. Gene expression levels of mitogen activated protein kinase interacting protein in two wild oyster populations (Green Hill Pond and Blue Bill Cove) and a proven disease resistant line (Rutgers).

Work Planned

Future work includes continual monitoring of our oysters deployed in Edgartown Great Pond. This will include documenting growth, survival, P. marinus prevalence and environmental conditions. Proven disease resistant strains from Rutgers University will also be deployed during the summer of 2006 to compare performance. Gene annotation is continuing with our Expressed Sequence Tag project on Crassostrea and over the next year, selected genes will be characterized in the oysters from our grow-out site in Edgartown Great Pond. Another major effort related to genetic characterization will include analyzing the differences in hemocyte gene expression patterns from a recently completed experiment conducted at the NMFS in Milford, CT by Gary Wikfors and Inke Sunila (participant on current project). Oysters from Connecticut with Dermo and oysters (C. virginica) from Washington State without Dermo, were subjected to mechanical stress. Hemocyte samples were harvested from each oyster and gene expression patterns will be characterized to better understand the relationship of immune function, disease and stress.

Impacts

While the results of our ongoing research have not been directly transferred to industry at this point, our data does suggest that shellfish farmers will be able to realize improved survival with local broodstock that has experienced persistent disease pressure. In addition, our gene expression data can be integrated into broodstock selection programs. These results would not only be beneficial to the oyster industry but could likely be used in developing superior broodstock in other shellfish.

Support

NRAC-USDA Funding	Matching Support	Total Support	
Year 1	$56,962	$13,979	$70,941
Year 2	$71,542	$13,979	$85,503
TOTAL	$128,486	$27,959	$156,445

Publications, manuscripts, or paper presented

Roberts, SB. 2006. Genomic approaches in characterizing shellfish disease: interrelationships between animal, human and ecosystem health. Cummings School of Veterinary Medicine at Tufts University, Annual Symposium: Marine and Aquatic Medicine & Conservation. North Grafton, MA. April 22, 2006.

Diner, E., Smolowitz, R., Gomez-Chiarri, M., Tammi, K., Leavitt, D., Roberts, S. 2006. Assessing disease tolerance in the eastern oyster using gene expression profiling. 26th Annual NOAA-NMFS Milford Aquaculture Symposium. Meridan, CT. February 28, 2006.

PROGRESS REPORT

PROJECT CODE: 05-3 SUBCONTRACT/ACCOUNT NO.: 557223

PROJECT TITLE: Effect of temperature on the infection of hard clams (*Mercenaria mercenaria*) by the protistan organisms, Quahog Parasite Unknown.

FUNDING LEVEL: $79,627 (Year 1) and $75,178 (Year 2)
Total: $154,805

PARTICIPANTS:

Funded
Roxanna Smolowitz, DVM, Marine Biological Laboratory
Dale Leavitt, Ph.D. Roger Williams University
Sandra Shumway, Ph.D., University of Connecticut
Gary Wikfors, Ph.D., Northeast Fisheries Center

Non-funded Participants
William Walton, Ph.D.,Cape Cod Cooperative Extension
Richard Kraus, Aquaculture Research Corporation
Leslie Sturmer Multi-county Extension Agent III; Sea Grant Extension Program

PROJECT OBJECTIVES:

1. Determine the occurrence and severity of QPX disease in the progeny of two strains of geographically distinct brood stock of hard clams originating from MA and FL at 7 different temperatures, sequentially over the two years in the laboratory, after:

a. No stress.
b. Stress caused by 4 months of low food availability and burrowing deprivation.
c. Acute, heat-induced stress.

2. Quantify hemocyte types/morphologies and functional ability, including phagocytic ability, in the hemolymph of 2 strains of hard clams at:

a. Three seasons/temperatures (spring, summer and fall) in both QPX-infected and uninfected clams cultured in the field.
b. Seven temperatures (with both QPX-infected and uninfected hard clams) in the laboratory temperature stress experiment.

ANTICIPATED BENEFITS:

1. Detailed data concerning temperatures at which QPX establishes infection in injected clams will be generated (expected to be within the range of 12-16°C) (Smolowitz).
2. Identification of the hemocyte types/morphology and functional abilities of hard clams hemocytes in relation to; a., the effects of temperature on the clams; b., differences between clams strains adapted to different climates; and c., causal effect of the above parameters on the development of QPX disease in hard clams (Wikfors).

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:

OBJECTIVE 1:

Determine the occurrence and severity of QPX disease in the progeny of two strains of geographically distinct brood stock of hard clams originating from MA and FL at 7 different temperatures, sequentially over the two years in the laboratory.

Clam Acquisition and Maintenance: Seed from Florida (2 hatcheries) has been obtained. Because of the hurricanes in Florida in 2004, seed of the size original requested in the grant was in short supply to culturists and researchers alike. However, with the help of Leslie Sturmer (U. of Florida multi-county Aquaculture Extension Agent), Tom McCrudden (Research Aquaculture, Stuart, FL) and Joe Weissman (Clams R Us, Vero Beach, FL)., approximately 28,000 hard clam seed (from Florida parent stock) of approximately 15 mm in shell height was purchased and shipped to the MBL. It was held at the Marine Resources Center (MRC) at the Marine Biological Laboratory until health certifications were finished, and until planting could be arranged. During this time, sea water that came into contact with the clams was treated to prevent spread of any disease. During the first 7 days, the static water used to cover the clams was chlorinated, heated to 60 °C, filtered to 2 μm, then discharged into the fresh water disposal. For the following 3 weeks, the ambient flowing water was diverted to a sump treated with chlorine and ozone. Hard Clam seed from Aquacultural Research Corporation (ARC) was also obtained and a health certification was conducted. One hundred animals from each group of seed were examined as part of the health certification. Since leases in Wellfleet recently were identified with QPX, and because of the problems associated with that new finding, it was no longer available as a negative QPX site. Therefore, with the guidance of Tom Marcotti (Town of Barnstable shellfish warden), we selected an intertidal site in Barnstable Harbor that to this date has not shown hard clams positive for QPX but in which clams have previously been cultured (Rendevous Bay). Control animals were planted in that site. The potentially positive QPX plots were located in the Town of Barnstable intertidal culture area adjacent to Scudders Lane. Both locations provide easy access to the plots at low tide. Three 7 ft. x 7 ft. plots were planted with clams from each clam origin at each location. An additional 3 plots were also planted with extra clams at each location. Density on all plots was 50 animals/sq. ft. Plots have been covered with conventional netting held down with rebar rods and staples since planting. The plots are being maintained (with one net change this spring) by a Residential Americore volunteer working with Dr. Walton and watched over by the Town of Barnstable shellfish department. Temperature is being continuously monitored with Onset Continuous Temperature Monitors at each site. Additionally, Dr. Walton has deployed a YSI probe in Barnstable Harbor that has a direct web site link (it is deployed only during the summer)

http://www.ysieconet.com/public/WebUI/Default.aspx?hidCustomerID=88).

Because of the high initial mortality of Florida clams in the MBL facility upon receival from Florida, clams from a New Jersey source were also ordered and planted in additional 7'x7' plots at both sites in Barnstable Harbor. Because of high initial mortality and the late acquisition of the clams (due to hurricanes in Florida) all clams not to be used in the initial laboratory experiment were planted out on the flats. Clams needed for the second set of laboratory experiments were collected from the uninfected control plots in November, 2005. These clams are being held at the MBL and have not been fed since retrieval. They are being used in the second set of laboratory experiments. Spring, 2006 examination of clam plots show that mortality is higher in the Florida clams than in NJ or MA clams. Moderate numbers of FL clams are lying on top of the plots underneath the netting. Whereas few clams are present on the tops of plots in the other strains of clams planted in the same areas.

Tank Set Up. Two locations at the MBL were established for the temperature exposure and all tank set up was completed in late September, 2005. A cold room at the MBL holds recirculating units set at 2, 10, 12 and 14°C (this space is donated for no fee by the MBL). The additional recirculating units, set at 16, 18 and 21°C were installed within the MRC (this space is normally fee based, but the fee has been partially waived for this project).

OBJECTIVE 1.a. No Stress

The first set of laboratory experiments began October 3, 2005. Six 12" x 12" plastic containers holding 1" of sand and approximately 2 liters of filtered sea water, were fitted with aerators and placed in a large tub containing recirculating, or static (depending on temperature required), fresh water that was either warmed or cooled to one of the temperatures noted above. 50 clams of either Florida or Massachusetts origin were placed in each of the 6 containers (3 of containers of each type) in each tub. Animals were acclimated for 2 weeks before the experiment began. After two weeks, 100 of each type of clam (FL and MA) were injected with 0.5 ml of QPX culture in a dilute media and 50 of each type of calm were injected with media diluted 1/10 with sterile sea water before placement in the containers. Samples were removed at 1, 2 and 3 months. Examination of injected clams from the first sample period showed some live QPX in tissues of several animals, but no progressive infections in any animals. The final two sets are still being evaluated. The experiment did not run for the 5 months as state in the grant because the animals were experiencing low to moderate mortality in the tanks, and the additional months of evaluation would not provide appropriate data.

OBJECTIVE 1.b. Stress caused by 4 months of low food availability and burrowing deprivation.

The second set of laboratory based experiments has begun. Animals were collected from plots at Rendezvous Point (the negative control site). Animals were starved for 7 months by holding in flow through sea water trays in the Marine Resources Center at the MBL. The animals will be injected and placed in the same containers as described above, however no sand will be added to the containers. Because Dr. Smolowitz's technician quit and the new technician is still in training (has only been working for 8 weeks), this second set of laboratory experiments has been delayed. However, it is expected that this experiment will last for only approximately 2 months since the clams are already experiencing some mortality in the holding trays. It is expected that the laboratory experiments will still meet appropriate deadlines as stated in the grant and based on the actual beginning date of the grant. Sampling for this objective will occur at 1 week, one month and two months. In addition, since we now have a real time QPX identification and quantification test method (developed by Dr. Steven Roberts at the MBL), we will sample the water column in the containers for the presence of QPX at the same time we sample the clams (additional work not stated in the grant) and evaluate them using the real time testing method.

OBJECTIVE 2:

Quantify hemocyte types/morphologies and functional ability, including phagocytic ability, in the hemolymph of 2 strains of hard clams.

a. Three seasons/temperatures (spring, summer and fall) in both QPX-infected and uninfected clams cultured in the field.

The second sample period for field planted animals was conducted in May, 2006. The water temperature goal was missed by 6 days. The temperature at collection was to be 13 C. However a severe and quick warming event shot the temperatures up to 18 C approximately 6 days before the sampling was scheduled. The animals therefore experienced 6 days of increasing temperatures (from 13 to 19 C before sampling occurred. All animals were sampled from the plots in the harbor the evening before processing. After retrieval, animals were quickly transported to temperature controlled containers where they were held till processing on the following day. The processing was set up in the Barnstable County Farmhouse. The county and Bill Walton donated the use of the building and the labor of 2 people for the day. In addition to Dr. Wikfors, and Helene Hergaret who conducted the analyses and Dr. Smolowitz and her technician, a total of 1 undergraduate student from U. CONN, 3 student aquatic interns spending the semester at the MBL (from Roger Williams University and Cape Cod Community College) and 1 Residential Americore representative spent the day bleeding 400, 2.5 cm shell height, hard clams to obtain hemolymph for FACS evaluation. In addition to collection of hemolymph from each animal, measurements and weight were taken as well as tissues for histopathological analysis. Wikfors and Hegaret brought the mobile FACS to the county farm house and ran each of the samples as they were collected. The next sampling period for the field work is scheduled for August 15/16, 2006. The temperature is expected to be at approximately 24 C at that time.

Immunological Analysis:

Analyses of hemolytic morphology and function were done on hemolymph extracted from the clams. Hemolymph was withdrawn with a needle and a 1-ml syringe from the adductor muscle of each individual clam, filtered with at 75- m mesh, and stored temporarily in an Eppendorf microcentrifuge tube on ice to retard cell clumping. Hemolymph of six clams was pooled together for each analysis. Procedures for characterization of clam hemocytes and for function (mortality, phagocytosis, aggregation and oxidative burst) were adapted from Hegaret et al. (2003 a and b) and from Lambert et al. (2003). We used a FACScan (BD Biosciences, San Jose, CA) flow cytometer for all hemocyte analyses.

Hematological parameters measured were: numbers of hemocytes detected during a set sampling time (an estimate of hemocyte counts per ml) as well as hemocyte characterization, in terms of size and internal complexity. The six immune functions measured were:

a.) Hemocyte viability, as a percentage of dead hemocytes

b.) Phagocytosis of fluorescent beads by hemocytes, which stimulates the engulfment of non-self particles

c.) Respiratory-burst response in hemocytes, that measures reactive oxygen species' potential to kill non-self particles previously engulfed by hemocyte and its ability to be induced by extra cellular products (ECP) of bacteria or inhibited with DPI.

d.) Adhesion capacity of the hemocytes.

e.) Percentage of apoptotic hemocytes.

Results to date:

Each hematological parameter was analyzed with Multifactor Analysis of Variance (MANOVA) with Site and Population as the two independent variables. The results indicate significant differences between the sites the clams are being grown in. Conversely, the origin of the population has not shown significantly affects on any hematological variable measured.

WORK PLANNED: Laboratory work as described in Objective 1. C. begin soon after 1. B. is completed. Field sampling and hemolymph evaluation of animals planted in Barnstable plots will occur again in the fall when the water temperature reaches approximately 13°C. Additionally, Dr. Smolowitz's laboratory is collecting samples of the water column above the plots of clams in both the infected and control locations in Barnstable Harbor to evaluate using a real time QPX testing method. That data will be integrated with the pathological evaluation of the animals to help answer the question: Is occurrence of QPX organisms in the water column predictive of the QPX infection status of the clams in the plot. The next sampling period for the field work portion of the grant is scheduled for August 15/16, 2006. The temperature is expected to be at approximately 25° C at that time (the highest temperature attained by the harbor waters). Water column samples for real time PCR evaluation are being taken monthly during the spring, summer and fall.

IMPACTS: Results of this work continue to be collected and evaluated. The local and regional culturists and the shell fish wardens are very interested in the project. The culturists continue to express interest in the project and appreciation for the action that the funding agency and researchers are putting into this project.

SUPPORT:

Year	Total NRAC Funding	Matching Funding	Total Funding
5-2005 to 7-2006	$154,805	$1,433	$156,238

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Barnstable County QPX Aquaculture Meetings. One was held in Wellfleet, MA and one at the Courthouse in the Town of Barnstable (during May, 2005). At these meetings the researchers met with town shellfish wardens, county representative and culturists to explain ongoing and planned work for areas affected by QPX. The work to be conducted in this grant was explained during those meetings.

Smolowitz, R. 2006. Objectives of the project on the effect **of** temperature on the infection of hard clams (*Mercenaria mercenaria*) by the protistan organisms, QPX. Presented at the National Shellfisheries Association conference Monterey, CA.

Dr. Wikfors demonstrating the use of the FACScan to clam breeders at the Barnstable Farm house.

ANNUAL PROGRESS REPORT

Project Code: 06-02-1 **Subcontract Number: Q169601**

PROJECT TITLE: Cross-Breeding and Field Trials of Disease-Resistant Eastern Oysters.

REPORTING PERIOD: June 15, 2006 to June 15, 2007

FUNDING LEVEL: $131,462

PARTICIPANTS:

Dr. Paul Rawson, University of Maine
Dr. Ximing Guo, Rutgers University
Dr. Roxanna Smolowitz, Marine Biological Laboratory
Dr. Marta Gomez-Chiarri, University of Rhode Island
Rick Karney, Martha's Vineyard Shellfish Group Dr.
Dale Leavitt, Roger Williams University
Dr. Bill Walton, Cape Cod Cooperative Extension
Inke Sunilla, CT Bureau of Aquaculture
Tessa Getchis, University of Connecticut
Dana Morse, Maine Sea Grant
Dr. Chris Davis, Pemaquid Oyster Co.

PROJECT OBJECTIVES:

Compare the growth and survival of oyster lines selected for disease resistance at grow-out sites in Maine, Massachusetts, Rhode Island and Connecticut via replicated common garden grow-out trials.

At the end of the 2 year grow-out trials, oyster lines demonstrating the highest yield (fastest growth and lowest incidence of disease) either at individual sites or across all sites will be set aside for future selective breeding. Non-proprietary strains will be kept in repositories split between the University of Maine and the Marine Biological Laboratory for future use by industry.

The project results will be presented at the annual NACE and Milford Aquaculture Seminar, at annual meetings of the National Shellfisheries Association, posted in a timely manner on a project-specific website, and disseminated to industry members via a Fact Sheets at the projects end.

ANTICIPATED BENEFITS:

This project will identify lines of oysters that perform well at a variety of sites in the region, particularly those sites where oyster diseases are endemic. It will also seek to develop lines of oysters that are resistant to multiple diseases. As such, the major product generated by this project will be a broodstock repository that will include lines that have demonstrated high performance, including disease resistance, at sites in New England. Should no single line be produced that performs the best at all sites tested, then the results of the proposed field trials will help growers in their choice of seed given their particular grow-out site characteristics (e.g. disease exposure).

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:

Our work to date has focused on producing 7 groups of putatively disease resistant oyster seed for deployment in field trials at sites throughout the New England states. In the fall and early winter of 2006 we began preparing hatchery facilities at the Darling Marine Center, Martha's Vineyard Shellfish Group and Noank Aquaculture Cooperative. In late January broods from two oyster stocks, the Rutgers University's NEH stock (resistant to the diseases Dermo and MSX) and the University of Maine's UMFS stock (resistant to JOD), as well as an NEH x UMFS hybrid line, were conditioned for spawning at the Darling Marine Center. Spawns were obtained from these three broodstocks in March of 2007 and used to construct 2 groups of pure line oyster seed (NEH, UMFS), a new generation of UMFS x NEH F1 hybrid oysters, and two backcross (F1 x UMFS and F1 x NEH) lines. Seed from these 5 lines have been set and have recently entered the nursery phase. Concurrently, two local strains from southern New England, ETGP and Clinton, were conditioned at the MVSG and Noank Aquaculture Cooperative hatcheries, respectively. Spawns from these latter strains were obtained in March 2007 and seed for each strain were set in April of 2007 and have since entered the nursery phase. We are on target with the hatchery and disease-resistant oyster seed producing component of our project.

During the spring of 2007 we have also been preparing the equipment necessary for the field trials in New Jersey (Guo), the Cape Cod Region (Lindell and colleagues) and Maine (Rawson and colleagues). The field component phase of the work will commence in June of 2007.

The MBL group under Scott Lindell is responsible for producing oyster seed from two local stocks of oysters and monitoring the performance of these stocks (with respect to growth and disease resistance) at several sites in New England. The MBL received 100 disease-resistant oyster broodstock from Edgartown Great Pond, Martha's Vineyard in mid-February. These were slowly warmed up and conditioned with cultured algae for 5 weeks until they were ripe. MBL staff transferred these oysters to Martha's Vineyard Shellfish Group and helped spawn them on March 19th. Currently, MVSG is culturing several million oyster seed from Edgartown Great Pond broodstock. The project only requires 40,000 seed that will be distributed to growers' field sites in early June. Inke Sunila of the Connecticut Department of Agriculture supplied disease-resistant oyster broodstock from Clinton, CT to the Noank Aquaculture Cooperative. According to Stuart Mattison, Noank's hatchery manager, these oysters have been conditioned and are ready to spawn. There was a problem with the quantity of algae being cultured in preparation for the spawn, and this has delayed the scheduled spawn by a week. The algae problem has been corrected and spawning was scheduled for late April. We are on target with the hatchery and disease-resistant oyster seed producing component of our project. The field component phase of the work will commence in June of 2007.

Under the proposed workplan, my lab is only involved in the grow-out portion of the experiment and not involved in hatchery production of seed oysters that will be tested during the grow-out phase of the project. Because the seed oysters for the field trials will not be ready for deployment until early June of 2007, our activities to date have been restricted to preparing the equipment necessary for the field trials in New Jersey.

WORK PLANNED:

We will begin side-by-side grow-out of the ETGP, Clinton, UMFS, and NEH lines at four sites in the Cape Cod region. We will also deploy seed from the UMFS, NEH, UMFS x F1 and backcross lines at a single site in New Jersey and four sites in Maine. These field deployments are expected to take place in June of 2007. Growers in each state have been selected based on their experience with disease and sites will include a range of tidal depths, salinities, bottom type and other environmental variables. Each of the growers at the field sites will receive approximately 18,000 seed (3,600 for each of the 5 strains) in a blind study and each strain will be represented in replicate ADPI bags or trays distributed across the site (n>3). We will monitor the performance of deployed lines (growth and survival) throughout the summer and fall of 2007. After oysters are overwintered they will be redeployed in March/April of 2008 and monitored through growth and survival of each strain in the spring, summer and fall of 2008. We will also test oysters for MSX disease prevalence and intensity in August and for Dermo disease prevalence and intensity in September and November of each year, particularly among oysters deployed in southern New England and New Jersey, using thioglycollate culture methods and histology as well as a recently real time PCR method of disease diagnosis for Dermo and MSX.

IMPACTS: - None to report, as yet.

Support:

YEAR	NRAC USDA Funding	OTHER SUPPORT					Total Support
		University	Industry	Other Federal	Other	Total	
Maine	$45,182	$40,976	$5,004			$45,980	$91,162
Rutgers	$ 15,535	$7,771	$29,907		$3,930	$7,771	$23,306
MBL	$70,745		$24,903		$3,930	$28,833	$99,758
TOTAL	$131,462	$48,747	$59,814		$7,860	$82,584	$214,046

Publications, Manuscripts, or Papers Presented:

Papers Presented

Rawson, Paul, Chris Davis, Bruce Barber, Bob Hawes & Scott Feindel. 2007. Oyster broodstock development in Maine: a cooperative effort between Maine's oyster industry and the University of Maine. Annual Meeting of the National Shellfisheries Association, San Antonio, TX, February 2007.