Oyster Research & Restoration in U.S. Coastal Waters

Developing Strategies for the Future

September 8-9, 2003
Annapolis, Maryland

Organized and supported by Maryland & Virginia Sea Grant Programs and the National Sea Grant Office

Sea Grant

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OYSTER RESEARCH AND RESTORATION IN U.S. COASTAL WATERS

WORKSHOP PURPOSE AND GOALS

The NOAA National Sea Grant College program has made a substantial commitment to research on numerous aspects of the domestic oyster fishery. This commitment represents a long-term, focused effort that has led to greater understanding of the biological, molecular genetic and ecological characteristics of these bivalves and the diseases that now impact them in U.S. coastal waters. The achievements of the Oyster Disease Research Program (ODRP) cover multiple areas, including the development of new tools for disease diagnosis, the successful breeding of disease resistant oyster strains, the development of new models of the interaction of disease and environmental factors and the development of a new understanding of the disease process at the cellular level. The Gulf Oyster Industry Program (GOIP) has extended this research to the Gulf of Mexico fishery and in addition has supported innovative research focused on numerous aspects of pathogens in oysters—including rapid detection and enumeration, new processing methods to ensure public health — while gaining an understanding of the impact of harmful algal species. A strong, collaborative interaction with industry partners has been a hallmark of this program.

In many states — particularly those in the Mid-Atlantic — efforts are being directed at developing far-reaching plans for restoration of oyster populations. The role of oysters as key elements in the complex trophic structure of coastal waters as well as their integral contribution to habitat are now well appreciated. Restoration efforts must combine a solid understanding of disease dynamics with large-scale environmental engineering focused on the rearing and release of hatchery-raised strains. In turn production strategies must be coupled to concerted efforts to rebuild vital oyster reef habitat. Such integrated efforts are the logical next steps in a process that builds upon the scientific foundations established over the past decade. In total, research directed to issues of ecology, disease and public health has provided basis upon which critical management decisions are now being made.

If NOAA Sea Grant is to remain an essential catalyst for these activities, it must build upon its past and continue to offer proactive scientific and policy insights for the future. To aid in this process, it is particularly important to synthesize what we have learned to date and develop consensus on how best to precede with new initiatives. A coherent strategy for future investments is needed. This meeting brings together representatives of the most relevant scientific, management, industry and public outreach communities with the following goals:

- Summarizing and synthesizing the status of oyster fisheries in the U.S.
- Sharing the most recent developments at the leading edge of oyster research with a focus on oyster disease, public health and processing as well as biological, ecological, aquaculture restoration issues.
- Detailing the most important and fruitful areas for research.

Our plenary sessions are built around contributions from recognized experts in their respective fields. The foundation developed therein will be used as a common starting point for workgroups that will be

charged with developing recommendations and strategies for future NOAA investments. Our intent is to build upon your diverse experience as key members of stakeholder communities (scientists, industry representatives, managers and the public) and initiate a process that will lead to consensus on fertile new directions for the federal investment. Your respective workgroups will be charged with developing strong position statements that reflect consensus on priorities. These will be shared in our final plenary session at the end of the meeting. The topics to be covered include:

- Oyster Fisheries Management and Restoration
- Genetics and Oyster Populations
- Frontiers of Disease Research
- Aquaculture and Hatchery Issues
- Public Health and Processing

Please note that the color of your badge will indicate the workgroup to which you have been assigned. Supporting, background materials for each of the workgroups is contained in this volume.

We greatly appreciate your willingness to participate with us. Our challenge over the next two days will be to evaluate the progress made nationwide to understand and manage oyster fisheries and to come to consensus to provide strong, community based recommendations. Your input and engagement are critical and we are grateful for the efforts you will make.

Prologue

Oyster Research and Restoration in U.S. Coastal Waters
Strategies for the Future

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Oyster Research and Restoration in U.S. Coastal Waters

Agenda

MONDAY, SEPTEMBER 8, 2003

REGISTRATION AND BREAKFAST: 7:30 a.m.

WELCOMING REMARKS: 8:15-8:45 a.m.
Jonathan Kramer, Maryland Sea Grant College Program
Invited Guests
VADM U.S. Navy Ret. C. Lautenbacher, Jr. Under Secretary of Commerce for Oceans and Atmosphere
Ronald Baird, Director, NOAA Sea Grant Program
Congressional Leaders

PLENARY SESSION I. Setting the Stage 8:45-10:00 a.m.

History of NOAA Sea Grant Oyster Research Programs
Jim McVey, NOAA National Sea Grant College Program

Background of NOAA Restoration Center, Oyster Restoration Projects
Kay McGraw, NOAA Restoration Center

Regional Updates on the Status of Oyster Populations

Gulf of Mexico
William S. Perret, Mississippi Department of Marine Resources

Pacific Northwest
Steve Bloomfield, South Sound Aquaculture

Chesapeake Bay
Roger Mann, Virginia Institute of Marine Science

BREAK: 10:00-10:20 a.m.

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BREAK: 10:00-10:20 a.m.

PLENARY SESSION II. The State of Oyster Disease and Environmental Research
10:20-12:30 p.m.

Current State of Knowledge on MSX Disease Caused by *Haplosporidium nelsoni*, and Priorities for Future Research
Eugene Burreson, Virginia Institute of Marine Science

Current State of Knowledge on Dermo Disease Caused by *Perkinsus marinus*
Jerome LaPeyre, Louisiana State University

Juvenile Oyster Disease: Progress and Directions in Research
Katherine Boettcher, University of Maine

Summer Mortality and *Crassostrea gigas*
Kim Reece, Virginia Institute of Marine Science

Molecular Technologies: Applications for Oyster Disease Research
Gerardo Vasta, Center of Marine Biotechnology, UMBI

The State of Oyster Disease and Environmental Research
Gary Rodrick, University of Florida

Genetics and Oyster Stocks
Standish Allen, Virginia Institute of Marine Science

The Crucial Ecological Role of Oysters in Chesapeake Bay
Roger Newell, University of Maryland Center for Environmental Science

LUNCH: 12:45-1:45 p.m.

The *Crassostrea ariakensis* issue
Mark Luckenbach, Virginia Institute of Marine Science

PLENARY SESSION III. Emerging Issues for Oyster Research: Regional Perspectives from Stakeholders
2:00 – 3:20

The Gulf's Viable Oyster Fishery
Mike Voisin, Gulf Oyster Industry Council

The Pacific Northwest Oyster Aquaculture
Dan Cheney, Pacific Shellfish Institute

The Mid-Atlantic
Mathilde Egge, Oyster Recovery Partnership

The Northeast
Dale Leavett, Roger Williams University

BREAK: 3:20-3:40 p.m.

WORKGROUP SESSIONS: 3:45-5:30

Oyster Fisheries Management and Restoration
Facilitator: Victor Kennedy, University of Maryland Center for Environmental Science

Genetics and Oyster Populations
Facilitator: Patrick Gaffney, University of Delaware Graduate College of Marine Studies

MSX and Dermo – Frontiers in Disease Research
Facilitator: William Fisher, EPA, National Health & Environmental Effects Research Lab

Public Health and Processing
Facilitator: John Supan, Louisiana Sea Grant College Program

Aquaculture and Hatchery Issues
Facilitator: Steve Bloomfield, South Sound Aquaculture

RECEPTION: 6:30-8:30 p.m.

TUESDAY, SEPTEMBER 9, 2003

BREAKFAST: 7:30 – 8:30 a.m.

CHARGE TO WORKGROUPS: 8:15 a.m.

WORKGROUP SESSIONS: 8:30-11:45 a.m.

BREAK: 10:30-10:45

LUNCH AND INITIAL REPORTS TO COMPARE PROGRESS: 12:00-1:30 p.m.

FINAL WORKGROUP SESSIONS: 1:30-3:00 p.m.

BREAK: 3:00-3:20

FINAL REPORT OUT IN PLENARY: 3:00-5:00 p.m.

ADJOURN

CONTEXT FOR OYSTER RESEARCH

National Sea Grant College Oyster Research Programs
Jim McVey, NOAA National Sea Grant College Program

For more than 30 years, NOAA Sea Grant has been supporting research in most coastal states on oyster disease, oyster biology and life history, production technology, post harvest treatments, industry management and economics, and ecological function. Broad-based research programs over the last ten years — in particular, the Oyster Disease Research Program and the Gulf Oyster Research Program —include strong education and extension components that have been providing scientific-based information for fishery managers, decision makers and the public. In addition, the NOAA Restoration Center has been supporting programs of coastal and marine habitat restoration to benefit the nation's fisheries and other trust resources. One goal of this meeting is to improve coordination among these programs as we develop research and restoration strategies for the future.

National Oyster Disease Research Program.

The National Oyster Disease Research Program (ODRP), which began in 1990 with oversight by the National Marine Fisheries Service and its Chesapeake Bay Office, is now administered through the National Sea Grant College Program. Congress funded the program after a series of meetings and workshops at the federal and state levels had clearly identified disease as one of the major factors in the decline of the mid-Atlantic oyster industry. Other coastal regions in the country including the Gulf Coast and the Pacific Coast were also experiencing significant oyster mortalities. To try and assist resource managers and oyster fisheries, ODRP research began focusing on a broad array of disease issues (1) optimum strategies for managing around diseases; (2) understanding the processes of parasitic infection; (3) improved understanding of the oyster's immune system; (4) hatchery techniques for producing disease-resistant strains; (5) molecular tools to better monitor the onset and presence of disease. To date, some $20 million has been invested on research; among the achievement have been the development of new tools for disease diagnosis and quantification; successful breeding of disease resistant oyster strains; development of new models on the interaction of disease and environmental factors; development of a new understanding of the disease process at the cellular level; and identification of several strains of known diseases and new species of shellfish parasites.

Gulf Oyster Industry Initiative

The Gulf Oyster Industry Program (GOIP) was created in 1999 as a result of efforts by the Gulf Oyster Industry Council, a broad cross-section of Gulf oyster industry leaders, state resource managers, and academic researchers. This group created a proposal for Congressional support of a Gulf Oyster Industry initiative that identified several key areas of research: (1) human pathogenic organisms; (2) consumer attitudes; (3) oyster diseases; (4) coastal restoration and freshwater diversions; (5) labor and mechanization in the oyster industry; (6) genetics and oyster hatchery technology; (7) harmful algal blooms and water pollution issues. About $4 million has been invested on these issue — among the programs achievements are the rapid detection and quantification of human pathogens in oysters; new post-harvest treatments and evaluation of those treatments to help assure public health; increased understanding of harmful algal species; an increased public education program targeted at immune compromised individuals to help reduce human health risks. The breadth of publications and presentations that have resulted from support of both ODRP and GOIP (see the accompanying list) may serve as a measure of how these programs have been contributing to the advancement of our understanding about oyster disease and issues related to the oyster industry and shellfish public health.

NOAA Restoration Center and Oyster Restoration Projects

Several federal and state agencies, private foundations and citizens groups have invested significantly in programs to rebuild oyster reefs, monitor oyster recruitment and survival on restored reefs, promote oyster gardening by waterfront owners, and create management programs for increasing oyster populations s in the Chesapeake Bay and other coastal areas. The role of oysters as key elements in the complex trophic structure of coastal waters, as well as their integral contribution to habitat, are now well appreciated. Research related to oyster ecology and disease has provided a foundation upon which critical management decisions are now being made. Restoration efforts must be coupled to concerted efforts to rebuild vital oyster reef habitat — such integrated efforts are important steps in a process that can build upon our improved scientific understanding about oyster disease and related issues that we have been gaining over the past decade.

The NOAA Restoration Center is the one office within NOAA solely devoted to restoring the coastal and marine habitats that support the nation's fisheries. Created in the early 1990s as an outgrowth of the *Exxon Valdez* oil spill, the NOAA Restoration Center provides restoration expertise and comprehensive restoration planning and implementation for coastal habitats and shellfish, including oysters. The Restoration Center has four main programs: the Damage Assessment and Restoration Program, the Community-based Restoration Program, the Coastal Wetlands, Planning, Protection and Restoration Act Program and the Restoration Research Program. Oyster restoration projects have been or are being implemented under all of these programs, though the majority are funded under the Community-based Restoration Program. The concept behind this particular program is to develop strong partnerships to accomplish meaningful, grassroots, habitat restoration activities that simultaneously promote a conservation ethic and wise stewardship of living marine resources. To date, the Restoration Center has funded about 40 oyster restoration projects that are in various stages of implementation. Several involve large-scale oyster reef restoration and others are smaller, for example., oyster gardening, the building of spawner sanctuaries, and the recycling of shells back into estuaries.

Among its achievements, the NOAA Restoration Center has helped enhance citizen awareness about the ecological value of oysters and oyster reefs and a commitment to restore them; increase oyster harvests in some areas; and furnish information about successful restoration techniques by different groups.

Non-indigenous Species in Restoration Efforts

One aspect of the restoration efforts in the mid-Atlantic has been the consideration of importing a non-native oyster *Crassostrea ariakensis*, to the Cheseapeake Bay and other coastal areas such as in North Carolina. This controversial issue has been a subject of discussion at several workshops and meetings in the Bay region and is now being considered by a committee of the National Academy of Science, which will make recommendations on its introduction to the Chesapeake late this summer. Since the purpose of this meeting is, in part, to determine the progress made thus far with the native oyster on the East and Gulf Coasts and to consider the future of oyster disease research and restoration, we have not organized a workgroup session on *C. ariakensis*; however, during the meeting, we will have an update on the current status of its potential introduction.

Structure of the Meeting

In this workshop our plan is to summarize the advances made through NOAA's Oyster Disease Research Program, the Gulf Oyster Industry Program and Cheseapeake Bay Oyster Restoration Program efforts. We will assess the health of natural populations of oysters around the country and discuss the emerging research issues of the future in a regional context. Breakout work groups will discuss and make recommendations on key issue areas including oyster fisheries management; genetics as it pertains to oyster research and recovery activities; the specifics of research needed for control of diseases; public health and post harvest processing; and aquaculture and hatchery issues.

PUBLICATIONS AND PRESENTATIONS FROM ODRP AND GOIP

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Allen, Jr., S.K. and T.J. Hilbish. 2001. Genetic considerations for hatchery-based restoration of oyster reefs. VIMS publication, 10 pp.

Anderson, R. S. and A. E. Beaven. 2000. Antimicrobial activity in cell-free hemolymph of oysters and mussels. J. Shellfish Res. 19: 641.

Anderson, R. S. and A. E. Beaven. 2001. Antibacterial activities of oyster (*Crassostrea virginica)* and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquat. Living Resour. 14: 343-349.

Anderson, R. S. and A. E. Beaven. 2001. A comparative study of anti-*Perkinsus marinus* activity in bivalve sera. J. Shellfish Res. 20: 1011-1017.

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Andrews, L.S., M. Jahncke, K. Mallikarjunan and C.D. Veal. 2002. Gamma irradiation processing to reduce the risk of Vibrio infections from raw oysters. Seafood Technology Division, Instititute of Food Technologists. 2002 IFT Annual Meeting, Anaheim, Ca. June.

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Brown, K.M., C. Ramcharan, B. Lezina, G. Peterson, and P. Banks. 2001. Novel deterrents to black drum predation on oyster leases. World Aquaculture Society Meeting at Lake Buena Visata, FL, p. 91 (abstract).

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Buchanan, J.T., C.G. Paniagua-Chavez, R.K. Cooper, and T.R. Tiersch. 2000. Research-scale Culture of Eastern Oysters. United States Chapter of the World Aquaculture Society Annual Meeting, New Orleans, Louisiana Annual Meeting, February 2-5. Book of Abstracts

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PLENARY SESSION ABSTRACTS

NOTES

Plenary Session Abstracts

Regional Updates on the Status of Oyster Populations

Gulf of Mexico, *William S. Perrett*

Pacific Northwest, *Steve Bloomfield*

Chesapeake Bay and Mid-Atlantic, *Roger Mann*

The State of Oyster Disease and Environmental Research

Pathogens Research Findings, *Gary Rodrick*

Summer Mortality on the Pacific Northwest, *Kim Reece*

Juvenile Oyster Disease: Progress and Directions in Research, *Katherine Boettcher*

Current State of Knowledge on MSX Disease caused by *Haplosporidium nelsoni*, and Priorities for Future Research, *Eugene Burreson*

Current State of Knowledge on Dermo Disease caused by *Perkinsus marinus* and Priorities for Future Research, *Jerome LaPeyre*

Molecular Technologies: Applications for Oyster Disease Research, *Gerardo Vasta*

Genetics and Oyster Stocks, *Standish Allen, Jr.*

The Crucial Ecological Role of Oysters in Chesapeake Bay, *Roger Newell*

GULF OF MEXICO: STATUS OF OYSTER POPULATIONS

William S. Perret, Mississippi Department of Marine Resources. Input for this presentation received from: Lance Robinson, Texas Parks and Wildlife Commission; Martin Bourgeois, Louisiana Department of Wildlife and Fisheries ; Scott Gordon, Mississippi Department of Marine Resources; Mark Van Hoose, Alabama Department of Conservation and Natural Resources; and Mark Berrigan, Florida Department of Agriculture and Consumer Services.

The eastern oyster, Crassostrea virginica, is distributed throughout the estuarine areas of the U.S. Gulf of Mexico. In some areas of the Gulf, oyster reefs are located in the states' territorial sea and even in the Gulf Exclusive Economic Zone (EEZ). Reefs are most abundant in shallow (under 40 feet) estuaries with salinities ranging from 5 to 20 ppt. Habitat is the most limiting factor controlling their abundance. This coupled with water quality and public health issues are this industry's greatest challenges.

Since oysters are primarily located in the estuarine areas of the states, they almost exclusively fall within the management jurisdiction of the individual states' natural resource agencies. For the Gulf these agencies are:

- Texas Parks and Wildlife Commission,
- Louisiana Wildlife and Fisheries Commission,
- Mississippi Department of Marine Resources
- Alabama Department of Conservation and Natural Resources, and
- Florida Fish and Wildlife Conservation Commission

Since these agencies are responsible for implementing rules, regulations, ordinances and/or statutes, they can and do have a dramatic effect on oyster management. Basic regulatory measures include, but are not limited to:

- Seasons,
- Daily bag limits,
- Size limits
- Gear restrictions,
- Harvest time restrictions,
- Leasing of water bottoms,
- Water quality monitoring,
- Data collection,
- Licensing and
- Enforcement

The Gulf of Mexico oyster fishery has a long and varied history. Production Gulfwide and statewide has fluctuated over time due primarily to habitat changes (including water quality) annually, seasonally and historically. In spite of this, Gulfwide oyster production has remained fairly stable and even increased in some geographic areas, unlike declining production in other areas of the county. Gulf production currently accounts for 59% of the oyster landings in the United States (during the period 1997-2001). This is up from approximately 36% for the period 1961-1965.

While Gulf production has remained fairly stable (with increases in some areas), the fishery is not without its problems. These include but are not limited to:

- Deterioration and loss of habitat (most serious).
- Development in estuarine areas.
- Modification of freshwater flow into estuaries for navigation, flood control and other purposes.
- Natural events (such as hurricanes and floods) and man-made activities (such as the removal of shells and cultch without returning sufficient amounts back to the reefs).
- Deteriorating water quality forces health agencies to close reefs when oysters become unsafe for human consumption.
- Oyster predators (drills – Thais haemastoma, stone crabs Menippe spp., black drum – Pogonias cromis), disease (Dermo – Perkinsus marinus), and harmful algae blooms, – (primarily Karenia brevis).

Data presented will include annual production figures by state, status of stocks by state, leased water bottom areas by state, public versus private lease production by state, factors affecting this production, and state activities relative to reef construction and rehabilitation, transplanting and relaying. Challenges facing the oyster resource, the industry and the management agencies will be discussed.

THE PACIFIC NORTHWEST — OYSTER AQUACULTURE

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The West Coast shellfish industry, tribal entities and fisheries agencies are faced with numerous opportunities and challenges with the start of the new millennium. Recent advances in shellfish genetics, hatchery, nursery and growout technology provide for the enhanced production of currently cultured species, the culture of new species and restoration of wild shellfish stocks. Coupled with these technological advancements are new international and domestic policies and programs calling for the support and development of robust, sustainable and environmentally sound aquaculture.

In light of these opportunities and challenges, the Pacific Shellfish Institute (PSI) encouraged shellfish growers, tribes, agencies, and the shellfish research community to establish goals for the year 2010 and the initiatives and research priorities necessary to achieve them. Prior to this effort, there were limited attempts to identify and prioritize shellfish research needs. Research institutions were criticized for not responding to public and private sector needs. Yet, with the limited input from industry, tribes and agencies that these institutions received, this criticism was probably not well deserved. Goals and priorities were first developed during a series of round-table discussions held in 1998-99. Drafts of these goals and priorities were also circulated to shellfish industry and tribal representatives, research institutions, granting entities and resource management agencies with a request for their assistance in completing the initiatives and research priorities. Input obtained from over 100 stakeholders was assembled and published in late 1999 in a report entitled *"North American West Coast Shellfish Industry 2010 Goals: Research & Initiative Priorities."* Beginning in early 2003, a PSI committee revisited key areas of *Goals 2010* to update and reprioritize research initiatives. Based on the existing 2010 document and committee input, the following general research needs, with oyster-specific examples, were identified:

- Genetics, genomics and improving domestication in oyster aquaculture – expand and continue genomic research, marker assisted selection, heterozygosity/homozygosity etc.; facilitate transition of breeding programs to the industry

- Culture of New Species – improvements in Kumamoto oyster, *Crassostrea sikamea*, broodstock and triploid production; breeding studies of the Suminoe Oyster, *Crassostrea ariakensis.*

- Ecology of oysters in estuarine systems – increase understanding of the ecological impacts associated with oyster culture and farming practices (eelgrass and shorebird mitigation, ESA listed salmonids); continue integrated approaches to nutrient/primary production/shellfish interactions

- Oyster pest/predator/prey control, monitoring and interactions – alternative and biological control of burrowing shrimp; identify, predict, and prioritize impacts of exotic pests, predators, macroalgae and seagrasses.

- Shellfish health management and disease/mortality prevention – increase emphasis on genetic approaches to disease management; risk evaluation and management methods for oyster diseases

- Processing: new methods and new products, and marketing of oyster products -- investigate the effects that HHP processing has on product shelf life, *Vibrio* bacteria, various toxic phytoplankton (PSP, domoic acid, DSP), viral pathogens (human) and spoilage microbes.

- Human health and oyster production – health issues associated with cadmium, dioxins, and Norwalk virus, plus on-going HAB concerns; changes in PSP and domoic acid in west coast estuaries.

- Oyster aquaculture education and promotion -- short-term training program including on-the-job training (i.e. algae culture methods, BMP outreach) with links to annual grower meetings or workshops (i.e. for regulatory training).

Today's presentation will briefly focus on these topics and other priority west coast oyster research areas. Researchers interested developing projects addressing these priority areas are encouraged to contact PSI, their state Sea Grant or aquaculture extension representative, local shellfish grower/harvester, tribal organization, and other appropriate parties.

Chesapeake Bay and the Mid-Atlantic — Oyster Population Estimation in the Chesapeake Bay and Strategies for Restoration

PRINCIPAL INVESTIGATORS: Roger Mann[1], Steve Jordan[2,3], Gary Smith[2], Kennedy Paynter[4], James Wesson[5], Mary Christman[4], Jessica Vanisko[2], Juliana Harding[1], Kelly Greenhawk[2], and Melissa Southworth[1]
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One of the principal goals of the Chesapeake Bay 2000 Agreement was a 10-fold increase in the biomass of the Bay oyster population by 2010. A collaborative between researchers in Maryland and Virginia was initiated to estimate baseline oyster population, and establish the monitoring, data management and data analysis frameworks for measuring progress toward the oyster restoration goal. Two estimators of the size of the oyster population in the Bay were developed as goals, these being the absolute number of oysters, and their biomass.

Both Maryland and Virginia support oyster fisheries. The Maryland fishery is almost exclusively a public access fishery. Virginia has an historical mix of a direct public access fishery and a private fishery based on leases of bay bottom. The leased private grounds can be planted with spat and harvested throughout the year once they reach the legal marketable size. Landings in Virginia illustrate a long-term decline and are at a level <1% of the historical high of the 1880's. Landings in Maryland have also declined rapidly from historic levels, then stabilized in the early 1990's, but are again in a state of decline. In both states the cumulative impacts of the diseases, MSX (*Haplsporidium nelsoni*) and Dermo (*Perkinsus marinus*) on harvest have been and continue to be substantial. Fishery dependent data contributes to population estimation in Maryland; however, the collapse of the fishery limits the use of such data in Virginia.

In Maryland, the Department of Natural Resources conducts an annual fall dredge survey at 43 sites that have been used to track changes in the oyster population as well as disease intensity and prevalence from 1990 to present. These sites are representative of the diverse types of oyster bars and have been supplemented with four additional sites that are closed to the fishery. Theycollectively comprise Maryland's sentinel sites and will be the focus of long term monitoring as part of the 2010 goal evaluation. Continuing field studies are focused on developing estimates of oyster dredge efficiencies that can be used to quantify the oyster population. In Virginia a combination of long term dredge data covering extensive areas has been combined with more focused patent tong surveys using stratified random sampling designs. The patent tong surveys provide a continuous data series for the James River for the period 1994- present. Long term data from the James using both dredge and tong data has been examined for application in developing a conversion function to estimate absolute abundance from dredge data. This conversion is the focus of continuing work. Virginia has identified 32 sentinel sites for long term monitoring of progress towards the 2010 goal, these being a combination of both historical sites and new sites in recent restoration areas. Identification and quantification of currently oyster habitat in both states remains an important aspect of this project in that these are estimated to be only modest fractions of historical habitat. The continued incorporation of refined habitat data through acoustic surveys will serve to improve population estimates.

Oyster density from fishery independent MD dredge surveys is estimated to range from 1.35 to 2.54 oysters per m^2, as estimated using the total population size extrapolated over an estimated area of high and low quality habitat available in the bay. In terms of biomass, it is reasonable to assume that the dry tissue weight of an average oyster is 1 g, so the total biomass would be between 5×10^6 and 6×10^8 kg. These estimates include all small (<76 mm) and market (>76 mm) oysters, but do not include spat. Estimates of the standing stock of market oysters in 2000 were 702,000 bushels for Maryland. Using fishery independent and dependent surveys, the 1994 baseline population estimates range from 0.29 to 1.00×10^9 oysters, with a mean of 0.64×10^9 oysters in Maryland. In Virginia, intensive patent tong surveys in the James River between 1993 and 2000 indicate a fairly stable population of 4.41 and 6.30×10^8 oysters with a current standing stock of approximately 365,000 bushels. Depending on location market oysters vary from <1% to 42% of the standing stock by number. Extrapolation of

dredge data for larger spatial areas in other rivers and regions in the Virginia portion of the Bay contribute to grand total estimates for the Virginia productive bottoms that vary from 5.31 x 10^9 to 6.00 x 10^{11} oysters. Present productive oyster ground in Virginia is well below the total surveyed by Baylor. The current calculations, which are arguably very optimistic, use only the modest values for high and moderate potential bottom area, as described by Haven et al. (1981); together they constitute only 24.85% of the total Baylor area within the Virginia portion of the Bay. Sub-market (<76mm) size oysters constitute a mean of 79% of the total (spat not included) for the period 1993-2001 in Virginia populations, a much higher percentage than in Maryland. Consequently biomass estimates for oysters in Virginia, which vary from 2.16 x 10^6 and 2.45 x 10^8 kg, are comparable to those for Maryland even though Virginia has an estimated higher population.

Young of the year recruitment, commonly termed spatfall, is assessed in both states by fall dredge surveys. In addition, Virginia employs shell string surveys. The Maryland Spat Index follows recruitment at the 43 sentinel stations for a period of over 60 years. The majority of years since 1986 show values in excess of the long-term median, indeed six years are above the 75% quartile; however, the past three years are generally between the 25% quartile and median. The comparable Virginia Spat index calculated for the past 16 years for 5 stations in the James River shows slightly higher median and quartile values than the Maryland long term record. VA indices within the past decade have generally remained between the 25% and 75% quartile. Both Maryland and Virginia enjoyed high spat indices in 1991, but the 1997 high value in Maryland was not reflected in Virginia where the lowest index in the 16-year record was recorded. The diseases, MSX (*Haplospridium nelsoni*) and Dermo (*Perkinsus marinus*) remain as threats to oyster populations in both states, and a substantial portion of natural mortality is directly attributable to one or both diseases. VIMS has maintained a disease monitoring program for MSX since 1960. A general trend of increasing maximum prevalence is observed over the 40-year period with intervals of decreased activity in the early 1970s, 1980s, and 1990s. Recent years have been characterized by very high maximal values. Maryland, generally observing lower salinity than Virginia across its 43 sentinel sites, has recorded considerably lower MSX prevalence since 1991, although this has notably increased above the average in the 2000-2002 period. The cumulative impacts of disease on long-term population trends are evident in recent analysis of stock assessment in the Maryland region of the bay. In the low salinity (<12 ppt) zone populations are moderate and stable in the face of limited disease pressure (MSX is rare and *Perkinsus* only in low intensity). In the mid salinity zone (12-14 ppt) a declining population from 1985 through 1994 was followed by a modest recovery in 1995-1999. By contrast no recovery has been observed in the high salinity zone (>14 ppt) where MSX in enzootic and *Perkinsus* causes consistently high mortality. The Virginia oyster resource is all predominantly in the > 14ppt region. Over the 1991-2001 period Maryland surveys have recorded sustained prevalence of *Perkinsus,* a marked increase in mean intensity in 2000-2002, and mortality in excess of 30%. Comparable data for Virginia have been collected along a salinity gradient in the James River from Deep Water Shoal to Wreck Shoal in a downstream direction.

Both Maryland and Virginia record their survey data as oyster per unit area, and size frequency as 5mm size classes. Size frequency data is being used in a length-based analysis of individual oyster growth. Oyster growth provides an essential link between spat settlement and the fishable stock. In order to evaluate potential oyster management options, it is essential to understand population dynamics and quantify parameter such as growth, natural mortality (M), fishing mortality (F), and recruitment (R). Additionally, size frequency data will be used to estimate M in Virginia populations, where F is negligible and in Maryland within fishery closures. These data can then be compared to box (articulated oyster shells) counts that comprise present annual mortality estimates in Maryland, but are believed to be biased high due to dead oyster shells that fail to disarticulate within a year. Refined oyster population parameters can be used within a framework to examine the efficacy of various restoration and management options. In stable populations knowledge of M can allow prediction of year strength recruitment in older year classes with the option to effect area management wherein submarket size class strength, after debiting for M, can facilitate prediction of harvestable stock (F, fishery mortality) for the subsequent year. The only stable population in Virginia, that is a population with minimal disease mortality, is in the low salinity sanctuary of the upper James River. Estimates for mean annual M values for 1, 2, 3 and 4 year olds are 0.16, 0.19, 0.55 and 0.14 respectively based on transformation of size demography to age demography using unpublished

growth data and annual survey data. However, extensive analysis of historical survey data for sub market and market oysters in concert with fishery landings suggest that submarket abundance is not a statistically robust predictor of fishery landings in subsequent years. Why is this? It is because consistent heavy disease pressure has effectively reduced F to near zero values, while M is both spatially and temporally variable depending on salinity and temperature as promoters of disease pressure – although disease is not the only contributor to high values of M. A collective failure of surveys, population dynamics and disease dynamics studies to date has been our inability to formulate estimates of M (by size and age class), F and R in time and location specific manners in order to develop and implement rebuilding plans in the manner employed by the regional fishery management councils. DEVELOPMENT OF ROBUST ESTIMATES OF M AND STRATEGIES TO LOWER M AND THUS FACILITATE REBUILDING THROUGH INCREASING R MUST BE THE COLLECTIVE HIGHEST PRIORITY OF BOTH POPULATION DYNAMICS AND DISEASE RESEARCH. Only through this strategy will there be any progress towards the 2010 goal. Further, we must all be aware that this highly visible barometer of restoration is also a commentary on our ability as a research community to respond to a defined biological problem.

Future management would benefit from definition of biological reference points, including over-fishing. A broader goal might also include developing a Total Allowable Catch (TAC) for Maryland alone, Maryland and Virginia together as part of a bi-state fishery, Virginia alone, and defined management zones based on salinity as a proxy for disease pressure. Once the above have been completed, evaluation of alternative reference points might provide new options for fishery management in concert with rebuilding to the 2010 goal. Finally, recognizing the unique value of shell as a habitat limiting resource for oysters, it is critical to improving R, a cost - benefit analysis of various methods of use of the shell resource is recommended. The suggested analysis will provide a framework for evaluation of introduction of oysters with lowered disease susceptibility on the genetic composition of the population in the Bay, with the long-term impact on estimation of M in the presence of continued disease challenge. Long-term data provide the basis to develop population models, examine possible stock recruit relationships, and examine the sensitivity of management options to variation in R, M and F.

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THE STATE OF OYSTER DISEASE

PATHOGENS RESEARCH FINDINGS

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Public and regulatory concern for persistent illnesses due to consumption of raw oysters, particularly for consumers at-risk due to compromised health, has culminated federal mandates to advance use of post harvest treatments (PHT) that could reduce the risk. These mandates specify measures in terms of actual amounts of production to be processed by new PHT increasing annually through the next five years (2002-2006). Failure to comply could result in more stringent regulatory consequences. New and innovative PHT methods that will not impose significant costs and training requirements and lead to market success are critically needed by the oyster industry.

SUMMER MORTALITY OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*, ALONG THE WEST COAST OF THE U.S.: IS OYSTER HERPES VIRUS ASSOCIATED WITH LOSSES OF SEED?

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Mortality of the Pacific oyster, *Crassostrea gigas*, has occurred in the Pacific Northwest and Japan since the mid 1950's and has only recently affected oysters in California beginning in 1993. Multiple stressors have been associated with these mortality events. In an attempt to alleviate the >50 annual oyster mortality observed in California and variable losses in Washington state, we examined the interaction between survivorship, growth and stress response of family lines from the Molluscan Broodstock Program (MBP) of Oregon State University, and planting time and height, and selected environmental parameters. Three oyster families were each planted during Fall 1999-2001 and Spring 2000-2002 at 2-3 sites in California, 3 sites in Washington (Spring only), and 1 site in Oregon (Spring 2002 only). Fall plants survived significantly more than did oysters planted in the spring ($p<0.05$) in California. In addition, two families (one commercial strain and MBP family 10-115) outperformed MBP family 10-116 ($p<0.001$) at all locations. During the study period inter-annual variation in phytoplankton community structure was more pronounced than spatial variation. Distinct seasonal patterns of community structure also emerged and were typical of temperate estuarine communities. While suspected harmful algal species were present throughout the study period, phytoplankton did not appear to be directly involved in oyster mortalities. However, in 2000 California mortality coincided with a *Gymnodinium sanguineum* bloom, while 2001 and 2002 mortalities were not associated with any phytoplankton bloom. Extreme temperature and dissolved oxygen fluctuations were repeatedly associated with oyster mortalities at the Washington and California study sites.

At the Tomales Bay, California site during 2002 oyster herpes virus was found infecting C. g/gas seed with prevalence ranging between 3.3 and 43. Oyster herpes virus has been reported from various species of oyster larvae and juveniles in French hatcheries in 1992 and subsequently claimed to be associated with high mortality rates, and "...abnormal mortality and morbidity." At the Tomales Bay site there was some correlation between mortality and herpes virus prevalence in the families, although this potential link will clearly require further investigation and surveillance. We are currently working on an east coast/west coast collaborative project examining Eastern {*Crassostrea virginica*}, Pacific (C. g/gas) and Suminoe (*C. ariakensis*) oysters for presence of the herpes virus using molecular and histological methods. During 2002 we screened spat and larvae from Maine to Louisiana along the US east and Gulf coasts, and along the US west coast in Washington and California. The purpose of this project is to determine if the virus is present in oysters in the United States and our findings to date have indicated that most U.S. stock of oyster larvae and spat are free of oyster herpes virus. Therefore, it is essential to further examine the significance of this positive finding in Tomales Bay. There has been some international resistance to listing the oyster herpes virus disease with the Organization Internationale Epizooties in 1995 (OIE, an international advisory organization) as a Notifiable disease (disease of highest significance that should be geographically contained) with the argument that the virus is widespread in oysters throughout the world. This project was initiated to further evaluate whether or not OIE listing or other management steps are needed for either exclusion or containment of the disease in North America.

We are employing molecular and histological assays for detection of the herpes virus. Sequence information and positive control material were provided to us by collaborators from IFREMER (T. Renault). The molecular methods were validated and initial U.S. collected field samples were examined during 2002. We are using both standard and nested PCR assays to ensure that we maximize the chance of detecting any strain variants of the virus. Selected samples are also evaluated by histology and in situ hybridization with DNA probes to localize and verify infection of oyster tissue. The positive PCR result from California indicats that it is of critical time

importance to establish the nature and magnitude of this threat to U.S. producers of oysters by answering the following questions: (1) Does the virus exist in and infect oysters in various regions of the United States and, (2) in locations in which it does exist, does the herpes-like virus constitute a significant risk to U.S. fishery resources? Whether a difference in prevalence reflects differential susceptibility of different oyster stocks needs to be assessed.. This information is needed to make appropriate recommendations regarding management of the virus and to take steps to prevent its spread, if oyster herpes viruses are found to be highly localized in occurrence in the U.S., and of significance to the oysters' health.

JUVENILE OYSTER DISEASE: PROGRESS AND DIRECTIONS IN RESEARCH

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It has been almost fifteen years since juvenile oyster disease emerged as a serious problem in the Northeast (especially in Maine and New York). Annual epizootics affect first-year seed during the nursery growout phase of commercial culture, and may kill more than 90% of a crop within a period of only a few weeks. Typically, signs such as uneven valve margins, proteinaceous shell deposits (conchiolin), and general emaciation immediately precede the onset of mortalities. No other species besides the Eastern oyster (*Crassostrea virginica*) is affected, and smaller animals (less than 25 mm shell height) are most vulnerable.

The etiological agent is believed to be a species of marine alpha-proteobacterium, which on the basis of phenotypic and phylogenetic analyses, represents a new taxon within the *Roseobacter* clade. This bacterium (proposed designation *Roseimarina crassostreae*, gen. nov, sp. nov.) is present as an almost pure culture on the tissue surfaces of affected animals, and has been associated with all documented epizootics in Maine since 1997. Last year, isolates were also recovered from two groups of animals that were survivors of JOD-epizootics in New York. Mortalities have been induced by experimental exposure to the bacterium, and the presence of *Roseimarina* in affected animals was confirmed by microbiological analyses. Experiments with laboratory held animals indicate that colonization by *Roseimarina* interferes with the ability of the animal to filter-feed, and that death results from some combination of starvation and/or bacterial toxin.

Low (< 5% cumulative) mortalities were observed in a population of fairly large animals (greater than 25 mm) in 2002. Still, conchiolin deposits were observed in 9% of oysters, and these animals were found to be extensively colonized by *Roseimarina.* The soft body tissues in oysters with conchiolin were still in good to fair condition, thus, JOD did not seem to be progressing beyond the initial stages. This was the first extensive sampling of (and subsequent recovery of *Roseimarina* from) larger animals with JOD signs but in the absence of significant mortalities.

The range of JOD is expanding, but for largely unknown reasons. (Some introductions are, however, clearly the result of transfer of infected seed). Just recently, the discovery of small variations in a DNA region between two ribosomal genes (the internal transcribed spacer, or ITS) has provided the basis for the first epidemiological studies of JOD. Restriction-digest analyses of PCR-amplification products revealed two genotypes: the first (GT1) was associated with JOD in Maine in 1997 and 1998, and a second (GT2) was recovered from all examined Maine epizootics in 2000, 2001, and 2002. Representatives of both genotypes were discovered among the 2002 New York isolates, and these regions are being sequenced to determine overall percent similarity to the Maine strains.

A PCR-diagnostic assay based on amplification of the ITS region is being developed in our lab. The specificity of the assay has been confirmed, and amplifications are successful with cells taken directly from culture plates. There are, however, still some problems with assay sensitivity using samples taken directly from animals (i.e. before recovery in culture). It appears that unknown factors in mucous or other extracellular products of oyster tissue are interfering with amplification. We are exploring several strategies for dealing with these contaminating substances.

Efforts are also directed toward the production of immunological reagents for detection of the pathogen. A polyclonal antibody has been produced in chickens from a 'cocktail' of *Roseimarina* cells (in various physiological states grown on solid and liquid media). In agglutination tests, the affinity-purified antibody shows strong reactivity to cells of *Roseimarina*, and no cross-reactivity with the most closely related bacterial species (*Roseovarius tolerans*). In tests of isolates obtained from 2002 epizootics in Maine and New York, a 100% correlation was observed between those isolates determined to be *Roseimarina* by the PCR-assay and those agglutinated by the antibody. Further, no agglutination has been observed with cells from colonies displaying morphologies similar to *Roseimarina* but which tested negative in the PCR-assay. Western blots will be performed to identify the specific proteins in *Roseimarina* which are recognized by the sera. Important

applications include a slide-based indirect fluorescent antibody test (IFAT) and in situ detection procedures to study host-pathogen interactions.

Very little is known about the process by which *Roseimarina* colonize and persist on surfaces of oyster tissues. Despite being in near constant contact with the ambient seawater, the outer tissue surfaces of healthy oysters remain essentially free of any attached bacteria. Yet, clearly *Roseimarina* can circumvent host defenses and become established on such tissues. We are currently investigating the hypothesis that oyster hemocytes are not always able to: (i) recognize or, (ii) phagocytose and destroy *Roseimarina* colonists. (Because oysters lack an antibody-based immune system, these cells form the primary line of immunological defense). Assays that measure the degree of hemocyte immunocompetence are being performed in both microtiter plates (where a reduction of a dye correlates with cell viability), and with standard-plate count techniques. We hope to define those conditions which are optimal for *C. virginica* to defend itself against *Roseimarina*, and use such information for the development of targeted prevention and/or remedial measures.

Of course, growers are primarily in need of options to help manage the risk of JOD. Previous work at the University of Maine demonstrated the clear relationship between bigger oysters and higher survival rates. These results led to the practice of early deployment of oyster seed such that most reach the 'size refuge' by the time epizootics typically occur (late Summer to early Fall). Still, for growers who conduct operations on enzootic systems (such as Maine's Damariscotta River), production becomes limited to a short time in the best growing season. An alternative solution may be that of 'biocontrol'. In 1998, a bacterium was discovered to be abundant in oysters which were apparently immune to JOD. We hypothesized that this bacterium might be a useful probiotic, and an experiment was conducted last year (in cooperation with a commercial grower) to evaluate its effectiveness. The results are inconclusive because there was a minimal natural impact of JOD, but they are nonetheless encouraging. Specifically, although control oysters did not suffer high mortalities, they did exhibit signs of JOD (notably conchiolin deposition). Such signs were absent in animals treated with the potential probiotic.

In much of this we are largely dependent upon the cooperation of oyster growers (one reports losing 50% of his production each year to JOD). However, the true impact and range of JOD is really not known, due to the reluctance of growers to report outbreaks. We've also found that during years of minimal mortality, JOD may be misdiagnosed (e,g, dismissed as 'normal' losses). Thus, one challenge is teaching growers to recognize JOD - in addition to advising them of the risks of seed transfer. It is likely to be more difficult to convince culturists to be more forthcoming and report suspected cases of JOD. They must first be persuaded of the importance of an accurate understanding of the range and site-specific losses caused by this disease.

Current State of Knowledge on MSX Disease caused by *Haplosporidium nelsoni*, and Priorities for Future Research

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Large-scale oyster mortality attributable to *Haplosporidium nelsoni* began in Delaware Bay in 1957 and in Chesapeake Bay in 1959. There is now good evidence from molecular studies that *H. nelsoni* is a natural parasite of the Pacific oyster, *Crassostrea gigas*, and was introduced to California and to the east coast of the United States. The exact timing or location of the introduction (or introductions) along the east coast has not been established, but the pathogen seems to have spread north and south from an initial epizootic in the middle Atlantic area.

The seasonal infection pattern of *H. nelsoni* is well established. New infections are acquired each year beginning in May and most infections occur in May, June and July; infection may continue through late summer in dry years. Infection intensity increases rapidly and oyster mortality usually peaks in August of the same year, within two to three months after infection. Intensity of infections acquired in late summer remains low through the winter and then increases in April and May as water temperature increases resulting in a second mortality peak in the spring.

Temperature and salinity tolerances of *H. nelsoni* are also well known. The parasite cannot survive at salinities of 10 ppt or below for more than 10 days. At salinities between 10 and 15 ppt the parasite can survive in oysters, but intensity usually remains low and little host mortality results. Above 15 ppt and temperatures above 20C the parasite multiplies rapidly and host mortality occurs with a few months of infection. There is some evidence from Delaware Bay that unusually cold winter temperatures result in lower prevalence and intensity of *H. nelsoni* the following summer.

Molecular diagnostic tools have been developed for *H. nelsoni*. Specific and sensitive PCR primers and DNA probes are available and have been used to provide evidence that *H. nelsoni* was introduced from the Pacific Ocean. Molecular diagnostic tools are currently being used in life cycle studies.

Mathematical models have been developed for population dynamics of *H. nelsoni* in oysters, and they accurately reflect actual data in areas not subject to high seasonal river flows. Models also suggest that an intermediate host is necessary in the life cycle to explain population dynamics of the parasite.
Oysters that demonstrate increased survival to *H. nelsoni* have been developed at Rutgers University and at VIMS through selective breeding. These oysters have been used successfully in New England and are being used in restoration and aquaculture efforts in Chesapeake Bay. There is also evidence that natural resistance to *H. nelsoni* has developed in oysters in Delaware Bay.

The four-year consecutive drought in the Mid-Atlantic from 1999 through 2002 has resulted in significant changes in the distribution of *H. nelsoni* in 2002. In Virginia, *H. nelsoni* was widely distributed and intensity was high, especially in upper tributaries. Prevalence at Horsehead Rock in the upper James River was a record high of 72%, and *H. nelsoni* occurred at Deep Water Shoal in the upper James River for the first time in history. In Maryland, 38 of 43 (88%) samples during 2002 had *H. nelsoni* infections and reflects a distribution record. Only five samples were free of *H. nelsoni*. The previous record high prevalence was 74% set in 1992. In 2002 *H. nelsoni* was found in the Chester River, Maryland for the first time in history.

However, the most significant range extension of *H. nelsoni* during 2002 was the epizootic oyster mortality in the Bras d'Or lake system in the Cape Breton area of Nova Scotia, Canada. *H. nelsoni* was not previously reported from Canada. Bras d'Or is a shallow, more or less isolated embayment that experiences warmer temperatures than surrounding locations, and 2002 was an unusually warm year, facilitating the establishment of *H. nelsoni*. It will be interesting to follow *H. nelsoni* abundance in this system when temperatures return to normal. *H. nelsoni*

is also present in Prince Edward Island, Canada, probably as a result of oyster transplantation from the Cape Breton area.

Although much has been learned about *H. nelsoni* biology over the last 40 years, critical gaps in our knowledge still exist. Most important is that the life cycle remains unsolved. Most scientists believe that an intermediate host is involved in the life cycle, but none has been identified to date. Life cycle studies have been hindered by a scarcity of spores, which are undoubtedly the infective stage to another host. Lack of knowledge on the life cycle has hindered interpretation of distributional changes, such as the recent range expansion into Canada, and has also rendered impossible controlled laboratory experiments to investigate pathological mechanisms and control measures. Controlled experiments have also been hindered by lack of a ready supply of the organism. Many advances in our knowledge of *Perkinsus marinus* were made possible because of successful continuous culture of the organism. Few attempts have been made to culture *H. nelsoni*, and none has been successful.

Future research efforts should concentrate on life cycle studies and obtaining continuous cultures of the organism. Both of these research avenues are relatively high risk, and expensive, but our understanding of *H. nelsoni* population dynamics and pathology cannot advance significantly until both are achieved. In addition, selective breeding, or other approaches, to increase tolerance of oysters to *H. nelsoni* should be continued.

CURRENT STATE OF KNOWLEDGE ON DERMO DISEASE CAUSED BY *PERKINSUS MARINUS*

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Dermo disease continues to cause extensive oyster mortalities along the Gulf of Mexico and Western mid-Atlantic coasts. Over the last decade, research on dermo has produced important information about the parasite and its interaction with eastern oysters. In particular, this research has provided evidence to further support some of the existing approaches used to control dermo, but has also revealed potential new strategies to control dermo more effectively. Diagnostic assays for *P. marinus* (including the FTM body burden assay considered the gold standard for *P. marinus* diagnostic) have been improved and new rapid, quantitative and specific assays for *P. marinus* have been developed. These diagnostic assays have lead to studies confirming *P. marinus* transmission dynamics models and revealed valuable information on environmental and host factors affecting the course of *P. marinus* infection in eastern oysters. We still lack knowledge of factors controlling parasite growth in oyster tissues during progression of infection and factors controlling parasite elimination during regression of infection. This knowledge will be key to understanding the underlying process in disease dynamics and for controlling the disease through more rational approaches. In vitro culture systems for *P. marinus* have been useful in ascertaining the effects of environmental factors (e.g., salinity, temperature, pH, O_2 and CO_2) on the parasite viability and growth, in characterizing its lipid metabolism, in identifying potential virulence factors such as proteases and in determining the population genetic structure of the parasite. Cultured parasites however have been shown to be less virulent than wild-type parasites hindering studies to fully understand the disease process. Determining the reasons for the loss of virulence of cultured parasites and restoring their virulence should be a research priority. *P. marinus* virulence factors can then be identified more easily and targeted to control dermo. Commercial inhibitors of *P. marinus* proteases for example have been shown to inhibit the growth of *P. marinus* in axenic culture and to decrease the number of parasites in infected hemocytes in vitro. Inhibitors of *P. marinus* proteases have also been recently purified from eastern oyster plasma along with other types of defense factors that are deleterious to *P. marinus*. The role these host defense factors play in increased dermo resistance recently observed in certain oyster stocks will need to be ascertained. Recently acquired information about *P. marinus* biology suggests promising new approaches to limit dermo mortalities, but also confirms the usefulness of a selective breeding program as a slow but proven method to increase dermo resistance in eastern oysters.

Molecular Technologies: Applications to Dermo Disease

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Introduction: During the past years, continued studies on the morphology, physiology, and biochemistry of *Perkinsus marinus*, and the effects of *P. marinus* on the oyster host, have resulted in substantial progress towards understanding the parasite's virulence and the environmental conditions that lead to epizootic outbreaks. The application of molecular approaches to Dermo disease in recent years, however, has resulted not only in the development of specific and sensitive diagnostic tools for *Perkinsus* spp, but also in a deeper understanding of various aspects of the parasite's biology and its interactions with the bivalve hosts. Although Fong *et al.* (Mar. Biol. Biotechnol., 2:346, 1993) amplified *Perkinsus marinus* rRNA genes by applying PCR to infected oyster hemocytes as early as 1993, the breakthrough that facilitated the comprehensive application of molecular approaches to the study of Dermo disease was the development by several research groups of *in vitro* culture techniques for Perkinsus spp. that enabled the production of large biomass of genetically homogeneous cells for isolation of nucleic acids and proteins. The unrestricted availability of *Perkinsus* spp. clonal cultures to the scientific community from a recognized public repository (ATCC, http://www.atcc.org) made it possible for several laboratories to apply molecular technologies to most of the species currently described.

Molecular diagnostics: The first molecular method for diagnosis of *P marinus* was a PCR-based assay targeted to the intergenic spacer (IGS) of the ribosomal RNA gene. This method was adapted into a semiquantitative format, and is sensitive enough to detect a single trophozoite in 30 mg of oyster tissue. This was later followed by a similar PCR-based method targeted to the internal transcribed spacer (ITS). The IGS proved to be a useful target for the development of strain-specific primers for *P. marinus* (types I and II), and more recently, for PCR-based dignosis of *P. andrewsi* and *P. atlanticus*. Further, the high conservation of IGS sequences close to the 5'end of the SSU rRNA in all *Perkinsus* spp. characterized at present enabled the design of primers that amplify all species currently available as holotype (hapantotype) cultures from ATCC (*P. marinus, P. andrewsi,* and *P. atlanticus*) and based on the sequence information available would also amplify *P. olseni*. This *Perkinsus* "genus-specific" PCR-based assay complements the species- and strain-specific assays developed earlier, and strengthen the detection of yet un-described *Perkinsus* spp. or those for which specific detection assays are not currently available. Quantitative PCR-based assay formats were later developed, and include competitive PCR (QC-PCR), real-time PCR (Taqman), and ELISA-PCR. The relevance of the species-/strain-specific molecular diagnostic methods over standard diagnostic methods (FTM, histology, etc) resides in that in some areas, such as Chesapeake Bay, multiple *Perkinsus* spp and strains are sympatric, and can be present simultaneously in the same individual oyster or clam.

Description of new species/strains: The characterization of rRNA genes from *Perkinsus* isolates from various host species, complemented with ultrastructural and gross morphology studies, led to the description of new *Perkinsus* spp. including *P. andrewsi* and *P. chesapeaki*. However, for *P. qugwadi*, a species that had been described as such based on morphological criteria, the later application of molecular approaches contributed to raise concerns about its true taxonomic placement within the genus *Perkinsus*. The application of molecular techniques has recently enabled the assessment of the genetic variability among isolates of *Perkinsus* spp. from various regions along the Atlantic and Gulf coasts of US.

Phylogenetic analysis: The phylogenetic affinities of *P. marinus* within the Alveolata has been controversial, and continues to generate great interest. Initially classified as a fungus, it was later placed within the Apicomplexa based on ultrastructure of the flagellated life stage. Molecular studies, however, suggested a closer affiliation to the dinoflagellates. The recent description of *Parvilucifera infectans*, a close relative of *Perkinsus* spp, lead to the establishment of the phylum Perkinsozoa, which would include both genera. An additional genus (*Cryptophagus*) has been recently added. The most recent phylogeny, based on tubulin, actin, and rRNA sequences, places the perkinsids as one of the earliest diverging group of the lineage leading to dinoflagellates. This phylogenetic position, however, has been inferred from limited available sequence information, and

additional genetic characterization of species from both clades, Dinozoa and Apicomplexa, will be required to support it.

Identification and structural/functional characterization of selected genes and their products: The availability of RNA and DNA from *Perkinsus* spp. cultures, and the use of PCR primers or hybridization probes based on consensus sequences from taxonomic related species, enabled the isolation and characterization of genes of interest for phylogenetic analysis, such as actins and tubulins, and for use in studies related to virulence and intracellular survival, such as superoxide dismutases (SODs) and iron transporters (Nramp). The application of molecular techniques has "subverted" the classical approach to structure/function studies, by avoiding the typical requirement of large quantities of the purified authentic protein. The bacterial expression of recombinant *P. marinus* SOD1 and SOD2 enabled the crystallization and resolution of both structures without prior purification of the authentic proteins. Gene complementation in heterologous systems (defective yeast mutants) led to the characterization of subcellular compartmentalization and biological function of the SOD gene products. Finally, this information facilitated both molecular and biochemical/physiological studies on the effect of stressors, such as reactive oxygen species and iron depletion, on gene expression, and cell viability. This has resulted in the identification of pathways the parasite uses to abrogate or avoid intracellular oxidative attack by the oyster hemocytes. For biomolecules synthesized by complex enzymatic pathways, such as carbohydrates and lipids, the implementation of classical biochemical approaches has been equally successful. The structural/functional characterization of *Perkinsus* proteins that may have critical roles as virulence factors, or in the parasite's metabolism, may lead to the rational design of chemotherapeutic agents for use in aquaculture settings.

Genomics: From the above it becomes clear that the knowledge of the complete gene repertoire of *P. marinus* could lead to the identification of additional genes required for parasite's virulence and, at the same time, suitable as targets for intervention. Therefore, it has become of great interest to elucidate the complete sequence of the *P. marinus* genome, and to examine gene expression under selected conditions by "expressed sequence tags" (ESTs). These comprehensive gene discovery programs in *P. marinus* are currently supported by NOAA/Sea Grant and NSF-USDA, and the information generated will be made available to the scientific community via public databases. ESTs generated from *P. marinus* propagated under various environmental conditions or exposed to host factors will result in unique gene expression profiles. Research on transcriptome differences among *Perkinsus* spp. will provide insight into their distinct virulence or responses to the host environment. These powerful approaches may be conceptualized as "ecogenomics " and "comparative genomics". Similar genomic studies are currently being carried out on the oyster elsewhere, and complementary technologies, including the construction of BAC libraries and microarrays, proteomics, and novel mathematical techniques, will contribute to examine oyster disease with a comprehensive, multidisciplinary approach.

The future: Implementation of molecular technologies such as those described above will rapidly increase *the* depth of knowledge of the parasite's biology, and facilitate future hypothesis-driven research on how environmental, host, and genetic factors modulate virulence in this ecologically and economically important pathogen. Furthermore, the vast information yielded by the application of genomic approaches, and the hypotheses-driven studies deriving from these, will make *Perkinsus* spp. excellent models for basic studies on fundamental processes that are common not only to parasites of mollusks and other invertebrates, but also parasites of medical and veterinary relevance. (Supported by awards from Sea Grant/NOAA, NRAC, NSF and USDA).

CURRENT AND FUTURE ROLES OF GENETICS IN OYSTER RESTORATION OR RECOVERY.

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ABSTRACT:
Speaking specifically to recovery of oysters in Chesapeake Bay, the practical role of genetics has been with assessment of and selection for disease resistance. Stock assessments have found genetic distinctions among populations. Field tests of distinct populations have uncovered important variation in disease resistance that is of practical value in selective breeding. Selective breeding programs originally intended for improvement of oysters for aquaculture have taken on a new life in oyster restoration, raising much broader questions concerning the genetic effects of stock enhancement.

Original programs for selection of disease resistance by Haskin (Rutgers) and Andrews (VIMS) were focused on survival to MSX-disease. The Haskin lines are extant in the form of the Haskin CROSBreed™ strains, which have had significant support from ODRP. Andrews original lines are extinct, but another line selected in Virginia was developed by VIMS, named Andrews DEBY™. DEBY oysters have been selected in an area with high intensity of both diseases and consequently, seem more Dermo-resistant than CROSBreed lines. CROSBreed and DEBY lines are appropriate for the mid-Atlantic, having been derived from oysters in Delaware Bay. Other lines have been developed from oysters north of Delaware Bay, the so-called NEH lines (NorthEast Haskin), also an offshoot of original lines developed by Haskin. NEH lines have been crossed with commercially derived JOD resistant strains in response to the episodic occurrence of this disease in areas north of Long Island.

Based on genetic differences among stocks along the eastern seaboard, testing programs have addressed variation in resistance to diseases. Stocks from the northeast are basically susceptible to both MSX- and Dermo-disease having seen little or none of it until recently. Northeast stocks also have markedly different reproductive strategies from mid-Atlantic ones. Stocks from Delaware Bay, although not clearly genetically distinct from other mid-Atlantic populations, have built up an innate resistance to MSX-disease probably attributable to the retention of larvae from survivors in the natural population. Circulation patterns are much more complex in the Chesapeake and movement of oysters is rampant. These factors probably contribute to the general lack of natural disease resistance there. Consequently, wild Chesapeake populations remain susceptible to both Dermo- and MSX-disease, which is the most serious impediment to oyster restoration. The genetically distinct stocks from the Gulf coast have developed innate resistance to Dermo-disease but are remarkably susceptible to MSX-disease. While not useful in the mid-Atlantic directly, germ plasm from Gulf stocks could play an important role in selection programs for dual disease resistance. Louisiana lines have been crossed into CROSBreed and DEBY lines.

Progress in selective breeding could be greatly enhanced through use of molecular markers in marker-assisted selection or pedigree analysis. Various classes of markers have been developed and applied to population genetics and pedigree analysis, but not mapping. These include allozymes, RFLP, microsatellites and SNPs. Different classes of markers have different utility. Microsatellites are broadly useful depending on the number of alleles. SNPs are more generally useful for mapping. As useful as mapping would be to obtain QTLs for disease resistance or other traits, mapping requires the generation of pedigreed families, in and of itself not a difficult task. However, there is an underappreciated logistical problem with raising pedigreed (inbred) families. Simply, there is no place to raise them out of the shadow of disease. Genetic studies could benefit from facilities that were designed for long-term isolation of oysters.

A new approach to reef restoration has seen the confluence of selective breeding, stock assessment, and molecular marker activities. Under the moniker

genetic rehabilitation, disease resistant stocks increasingly have been incorporated into outplantings of seed for oyster reefs. The notion arose almost by default. Reef building has been important in the Chesapeake for nearly 10 years. Stocking reefs with oysters has been popular since the mid-1990s. The notion to stock reefs with selectively bred, disease resistant seed came as a direct result of the release of CROSBreed oysters, and later DEBYs, in the early 90s. Now it seems that the welfare of reef restoration in general is pinned on the hopes of disease resistant stocks – at least in Virginia.

Genetic rehabilitation is a simple concept with dastardly complex logistics and assessment issues. Conceptually, disease resistant (or at least disease tolerant) oysters are created through selective breeding program(s) and released to hatcheries for production of seed which is nurtured to refuge size. Hatchery derived seed is planted on "incubator" (or "breeder") reefs where it has the opportunity to spawn producing a new crop of recruits on prepared, shelled areas. This reef-derived seed is then harvested and moved to other reefs in a continual transfer of seed hopefully enhanced for disease resistance. In genetic rehabilitation, molecular markers play a key role in assessment of recruitment success of enhanced stocks.

From the perspective of the selective breeding that gives rise to the disease resistant lines, there are some serious genetic questions concerning the most optimum approach for restoration versus aquaculture. For example, if disease resistance is additive, then there is merit to developing unique and specific lines with specific combining ability for both programs. If disease resistance shows pleiotropy or epistasis, a more productive approach might be selection for general combining ability for restoration, but not necessarily for aquaculture. Genetic dissection of these characters will be essential for both improving the rate of approach to disease resistance and understanding the prognosis for success in genetic rehabilitation.

Finally, non-native species are starting to crowd center stage. ODRP supported work with non-native species in the early 90's in studies on hybridization potential among *C. virginica*, *C. gigas*, and *C. ariakensis*. An important offshoot of this work – despite the failure of *C. virginica* to hybridize with the others – was the importation of stocks of *C. ariakensis*. These later became the basis of stocks used to assess the value of this species in Chesapeake Bay. The value seems high. Up to now, no field work on *C. ariakensis* has been funded by ODRP, but as the momentum for introduction grows, so does the need for answers concerning the ecological impact. Only some of these questions are genetic in nature. Later, however, if *C. ariakensis* is introduced, genetics will be important because of questions concerned with stock enhancement, domestication, creation of sterile stocks, or all the above.

THE CRUCIAL ECOLOGICAL ROLE OF OYSTERS IN CHESAPEAKE BAY

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ABSTRACT:
Suspension feeding bivalve molluscs serve to couple pelagic and benthic processes by filtering suspended particles from the water column and transferring undigested remains in their feces and pseudofeces to the sediment surface. This activity can be extremely important in regulating water column processes where bivalves are abundant in coastal waters and in seasons when water temperatures are warm enough to promote active feeding. Of all bivalve species worldwide, eastern oysters *(Crassostrea virginica)*, are among the most powerful in this regard because of their unusually high weight specific filtration rates (~7 to 10 L h^{-1} g^{-1} dry tissue weight at typical summer water temperatures of 25°C.) Adult eastern oysters are well adapted to living in estuaries, such as Chesapeake Bay, where inorganic particles comprise a large fraction of the seston because they can sort filtered particles prior to ingestion and reject less nutritious particles as pseudofeces.

This feeding activity enables large populations of oysters to reduce phytoplankton assemblages, thereby decreasing turbidity and increasing the amount of light that reaches the sediment surface. In this process, oysters exert "top-down" grazer control on phytoplankton production and extend the depth to which ecologically important benthic plants, such as seagrasses and benthic microalgae, can grow. Unfortunately, the extensive populations of eastern oysters that once dominated Chesapeake Bay are but a mere remnant of their original abundances. If oysters harvests can serve as an index of population abundance, the decline from more than 10 million bushels a year in Maryland in the late 19th century to some 2 million a year in 1985 to less than 100,000 in these last several years reflects the catastrophic state of the Chesapeake oyster stocks. It is likely that the loss of this keystone suspension feeder has had profound adverse effects on the ecology of Chesapeake Bay.

Newell (1988) estimated that it took eastern oysters less than a week to filter the entire water volume of Chesapeake Bay when oysters were highly abundant in the 1880's before stocks were commercially exploited. Today, oyster stocks are at an all time low due to a combination of ongoing oyster disease epizootics and destructive harvest practices reducing oyster reef habitat quality. One way to gauge the ecosystem changes that may result from this loss of oysters is that it now takes the oyster stocks in the Bay about one year to filter the water volume of the Bay. Furthermore, the loss of oyster reef substrate that various invertebrate and invertebrate organisms in the Bay once utilized for shelter and feeding has altered animal community composition.

Some critics of Newell's (1988) proposition that oysters once exerted top-down control on phytoplankton stocks have argued that oysters simply recycle inorganic nutrients rapidly back to the water column and hence there would not have been any long-lasting reduction in phytoplankton biomass. To help distinguish between these scenarios, Newell et al. (2002) explored in laboratory incubations changes in nitrogen fluxes and denitrification under anoxic and oxic conditions in response to loading by different amounts of phytoplankton cells, representing an experimental analog of oyster biodeposits. When organics were regenerated under aerobic conditions, typical of those associated with shallow water oyster habitats, coupled nitrification-denitrification was promoted, resulting in denitrification of ~20% of the total added nitrogen. In contrast under anoxic conditions, typical of current summertime conditions in main-stem Chesapeake Bay where phytoplankton is microbially degraded beneath the pycnocline, nitrogen was released solely as ammonium from the added organics. This study indicates that denitrification of particulate nitrogen remaining in the biodeposits of oysters will enhance nitrogen removal from Chesapeake Bay. Phosphorus remaining in their biodeposits can become buried and sequestered within the aerobic sediments. In summary, it is now apparent that sufficient numbers of eastern oysters can exert both "top-down" control by grazing on phytoplankton stocks and influence "bottom-up" nutrient control on phytoplankton production by changing nitrogen and phosphorus regeneration processes within the sediment. Thus, restoration of the once abundant stocks of oysters to Chesapeake Bay may be a crucial complement to other management

activities that seek to reduce phytoplankton production by curbing N and P inputs from point and non-point sources.

It is plausible that an ecosystem dominated by benthic primary production may develop in shallow waters when reduced turbidity associated with oyster feeding increases light penetration to a level that can sustain benthic microalgal production. These benthic microalgae compete with nitrifying bacteria for N regenerated from oyster biodeposits, thereby reducing or even precluding coupled nitrification-denitrification. Although these benthic microalgae are an important food source for many benthic animals, it means that nitrogen removal via denitrification will not be an important nitrogen removal pathway in the shallows.

Over the last four decades seagrass beds have either declined or disappeared throughout much of the Chesapeake Bay due to high water turbidity leading to reduced light availability for these benthic plants. We (Hood, Koch, and Newell) are developing a numerical model to explore the possible interactions between oyster and seagrass declines. Once complete, this will simulate the effects on seagrass growth of the interactions between wave-induced sediment resuspension, oyster filtration, and the direct influence of the physical structure of the oyster reef itself on wave action. Predictions from this model shows that under high wave height conditions the presence of oysters can reduce suspended sediment concentrations by nearly an order of magnitude, which significantly increases water clarity and the depth to which seagrasses can grow.

It is now widely believed that these ecological functions of eastern oyster populations are so vital that it is important to have extensive oyster populations in the estuaries along the Atlantic and Gulf coasts. Unfortunately, restoration activities in Chesapeake Bay over the last 5 y have largely been stymied by worsening Dermo and MSX epizootics. In Maryland, restoration primarily involves placing hatchery-reared spat in low salinity regions where a group of scientists, including me, expected that diseases would be less virulent over the long-term. This has proved to be an incorrect supposition as high salinities in recent years have allowed diseases to invade even these regions and kill oysters on many restored bars.

Recent incremental advances in the development of disease tolerant strains of oysters by Allen and coworkers means that in highly controlled aquaculture situations, where growth is rapid, oysters can now reach market size without appreciable disease mortality. Unfortunately, because of the much slower growth rates when growing on natural oyster bars, these strains are not yet sufficiently disease tolerant to survive for the extended period desirable to maximize ecological function for restoration projects. What is required is a highly disease tolerant strain of oyster that can be used for restocking oyster bars for ecological function and public harvest. It is likely that progeny from disease tolerant oysters on unharvested bars can help rebuild natural stocks, hence ultimately reducing reliance on hatchery production. I recommend that we rigorously evaluate recent research to determine if the development of a strain of oyster that is highly tolerant to MSX and Dermo is achievable in the next 5 to 10 y. If we believe it is attainable we should focus ODR funding efforts more strongly on the development of such a strain and put less emphasis on other ODR research activities.

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Newell, R.I.E, J.C.Cornwell and M. S.Owens. 2002. Influence of simulated bivalve biodeposition and microphytobenthos on sediment nitrogen dynamics: a laboratory study. Limnol. Oceanogr. 47: 1367-1379. Free download at http://aslo.org/lo/toc/vol_47/issue_5/1367.pdf

WORKGROUP 1

OYSTER FISHERIES MANAGEMENT AND RESTORATION

NOTES

WORKGROUP 1

OYSTER FISHERIES MANAGEMENT AND RESTORATION

Field and laboratory study of the process and dynamics of *Perkinsus marinus* in the eastern oyster, *C. virginica*. Eugene Burreson, Lisa Ragone Calvo, Christopher Dungan, Bob Roberson. Apr 1, 1994-Mar 31, 1995

Environmental effects on *Perkinsus marinus* infection rates, growth and survival among dermo-disease-free juvenile individuals planted at three salinity regimes in an enzootic Chesapeake Bay Oyster Recovery Area. George Abbe, Chris Dungan and Steve Jordan, Jan. 1, 2000-Dec. 31, 2001

In situ determination of *Perkinsus marinus* transmission dynamics in low salinity habitats: Implications for disease avoidance management strategies and oyster restoration. Kennedy Paynter, Eugene Burreson, Kimberly Reece, Lisa Ragone-Calvo, Oct. 1, 2001-Sept 31, 2003

Predicting time to critical levels of *Perkinsus marinus* in eastern oysters, *Crassostrea virginica*: A new tool for increasing oyster production. Sammy Ray, Thomas Soniat, Enrique Kortright, Sept 1, 1998-Aug. 31,2000

DermoWatch: A web-based approach for managing *Perkinsus marinus* disease of oysters. Sept. 1, 1999-Oct. 8, 2003

Development and management applications of a dual-disease (MSX and Dermo) model for Chesapeake Bay oyster populations. Eileen Hofmann, J.M. Klinck, E.N.Powell, S. E. Ford, S.J. Jordan, Oct 1, 1999-July 31, 2002

Modeling the effects of climate variablility on the prevalence and intensity of Dermo and MSX diseases in eastern oyster populations. Eileen Hofmann, J.M. Klinck, S. Ford, E. Powell, S. Jordan, E. Burreson, Oct. 1, 1999-July 3, 2002

Oyster predator-prey interactions: Roles of different predators, seasonality, spatial variation and deterrents. Kenneth Brown, Gary Peterson, Mike McDonough and Charles Ramcharan.

Oyster harvest efforts in Pre-Diversion Barataria Bay During a Decade of Salinity Shifts, 1990-99. Earl J. Melancon, Jr., Eric Swenson and Peter Vujnovich, Jr., 2001-2003.

Ground truthing hydroacoustic based estimates of oyster reef with commercial oyster dredging. Charles Wilson and Harry Roberts, December 1, 2000-March 31, 2003.

Video documentary on the rise and fall of the Chesapeake Bay oyster fishery. Michael W. Fincham and John Greer.

FUNDING PERIOD 4/1/94-3/31/95

PROJECT TITLE: Field and laboratory study of the process and dynamics of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*

PRINCIPAL INVESTIGATOR: Eugene M. Burreson and Lisa M. Ragone Calvo
AFFILIATION: Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062

CO-INVESTIGATORS and AFFILIATIONS: Christopher F. Dungan, Cooperative Oxford Laboratory, Maryland Department of Natural Resources, Oxford Maryland, Bob S. Roberson, Department of Microbiology, University of Maryland, College Park, MD 20742

PROJECT RESULTS:
This project represents the second year of a two-year study, which is the first study to systematically examine the seasonality of *P. marinus* infection acquisition in oysters in relation to water column abundance of *P. marinus* cells, oyster mortality, and temperature. The timing and magnitude of seasonal peaks in environmental abundances of *P. marinus* cells and local oyster mortalities at an upper (Tred Avon River, MD) and lower (York River, VA) Chesapeake Bay site were also contrasted. Lastly, a specific and sensitive immunoassay detection technique was employed to examine the process and site(s) of pathogen invasion into host oysters in both field and laboratory *P. marinus* exposures.

Uninfected sentinel oysters were naturally exposed to the parasite during two-week intervals throughout the course of the study to determine the periodicity and rates of parasite transmission. The timing and magnitude of disease-associated oyster mortalities in a local *P. marinus*-infected oyster population were estimated by monitoring a captive subset of the local oyster population. Flow cytometric immunodetection methods were employed to estimate the abundance of *P. marinus* cells in water samples collected three times each week.

In the lower York River, VA, environmental abundance of *P. marinus* cells, infection acquisition by sentinel oysters, and mortality of *P. marinus*-infected oysters varied seasonally. Distinct peaks of all three parameters occurred during the month of August, following maximal summer temperatures. Water column parasite cell abundances, infection pressure, and oyster mortalities decreased from summer maximums as temperatures decreased in September and October, and remained at "wintertime" low levels from October through the termination of the study in March. Counts of antibody-labeled cells ranged from 10 to 11,900 cells per liter, Strong and significant positive correlations were found between water column parasite cell abundance and temperature, water column parasite cell abundance and oyster mortality, oyster mortality and temperature, and oyster mortality and *P. marinus* prevalence in sentinel oysters.

In the Tred Avon River, MD, maximum abundances of *P. marinus* cells in the water column were also observed during August. Environmental cell abundance was significantly correlated with temperature, but not with local oyster mortality rates. Abundance levels overall were generally higher than at the York River site but were still of the same order of magnitude. Local oyster mortality at the upper Bay site occurred later in the summer and was much lower than at the York River site.

These results support the prevailing model of *P. marinus* transmission dynamics that maximum transmission rates are observed during periods of maximum *P. marinus*-associated host mortality. However, results also indicate that transmission can occur when host mortality is low or absent; so alternative mortality-independent dissemination mechanisms are likely. Results also suggest that atypically early summer oyster mortality from *Haplosporidium nelsoni* infection, at a time when infections of *P. marinus* are light, has a significant indirect influence on *P. marinus* transmission dynamics. Elimination of these hosts prior to late summer *P. marinus* infection intensification effectively reduces the overall number of *P. marinus* cells disseminated.

Finally, the results of *P. marinus* initial infection studies suggest that the digestive tract may not be the only, or even the primary, site of infection. The localization of many parasite cells in gill and mantle epithelia may be indicative of alternate pathogen entry routes.

IMPACTS and/or BENEFITS:
The project resulted in improved understanding of *P. marinus* transmission dynamics. Such understanding was key to the development of predictive models of *P. marinus* disease dynamics and this information has also been valuable in the development of disease avoidance strategies promoting enhanced management of oyster fisheries and oyster aquaculture.

PROJECT PUBLICATIONS:

Ragone Calvo, LM., C. F. Dungan, B. S. Roberson and E. M. Burreson. 2002. A systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in the lower Chesapeake Bay, Diseases of Aquatic Organisms. *Diseases of Aquatic Organisms. In Press.*

Ragone Calvo, L.M., R.L. Wetzel, E.M. Burreson. 2001. Development and verification of a model for the population dynamics of the protistan parasite *Perkinsus marinus* within its host, the eastern oyster, *Crassostrea virginica* in Chesapeake Bay. Journal of Shellfish Research 20(1): 231-241.

Burreson, E.M. and L. Ragone Calvo. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. Journal of Shellfish Research Special Publication. Journal of Shellfish Research. 15(1):17-34.

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Dungan, C.F., Hamilton, R.M. ; Burreson, E.M.; Ragone-Calvo, L.M. 1996. Identification of *Perkinsus marinus* portals of entry by histochemical immunoassays of challenged oysters. Journal of Shellfish Research 15 (2):500.

FUNDING PERIOD: Oct. 1, 2001 – Sept. 30, 2003

PROJECT TITLE: Environmental effects on *Perkinsus marinus* infection rates, growth and survival among dermo-disease-free juvenile oysters planted at three salinity regimes in an enzootic Chesapeake Bay Oyster Recovery Area (continued)

PRINCIPAL INVESTIGATOR: George Abbe
AFFILIATION: Academy of Natural Sciences Estuarine Research Center
CO-INVESTIGATORS and AFFILIATIONS: Chris Dungan and Steve Jordan, Sarbanes Cooperative Oxford Laboratory

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PROJECT RESULTS:

In 2002 specific pathogen-free (SPF) oysters were set on oyster shell and deployed along a salinity gradient in the Patuxent River, Maryland to investigate environmental effects of *Perkinsus marinus* on infection rates, growth and survival. Spat were deployed at the three original sites located on oyster bars and a new site at Sandgates (SG) located at least 1 km from natural bars for assay of *P. marinus* infections by the whole body burden technique which allowed an estimate of time to initial infection and subsequent progression of dermo disease. Additional oysters were monitored monthly for growth and mortality. Thirty oysters from the natural population at each site were also examined monthly by rectal tissue assay. During the first year, salinity at Holland Point (HP, upper river), Gatton (GAT, mid) and Town Creek (TC, lower) averaged 11.1, 13.0 and 14.4, respectively, but during the second year averaged 13.3, 15.8, and 16.9.

Spat at HP, GAT and TC grew 23, 34 and 27 mm, respectively, and survival was 95, 98 and 94% during the first year. During the second year, however, growth was slightly better at HP (21 mm) than at GAT (16mm) or TC (19mm), but mortalities began to accelerate in October 01 at TC, in June 02 at GAT and in August 02 at HP. By August 2002, mortalities at HP, GAT and TC were 60, 98 and 97%, respectively, and HP reached 97% 2 months later. Two discrete spat sets and deployments were made: May 2002 and September 2002. From the May deployment, SPF oysters placed at SG, remote from existing populations, acquired infections by day 27 (13% prevalence), as did juveniles deployed at TC (7%) and HP (3%). At all sites animals acquired *P. marinus* infections within 62 days, with prevalences of 10%, 63%, 43%, and 37% (TC - HP). By 91days post-deployment all sites, with the exception of TC, had infection prevalences greater than 90%, and these elevated prevalences continued through 127 days. At 91days TC prevalence remained low at 10%, but by 127 days it increased to 53%. In October prevalences declined at TC, GA, and SG (154 days), but all remained above zero into mid-November. SPF juveniles deployed in late September acquired *P. marinus* infections by 4 weeks at all sites, however prevalences were low and declined at 8 weeks, with infections detected then only at HP.

Mean intensity of dermo disease among feral populations (on a scale of 0-7) at HP, GAT and TC averaged 2.51, 2.72 and 2.79, respectively, during the first year and 2.65, 2.81, and 1.81 during the second. Mortalities were high on all three bars.

IMPACTS and/or BENEFITS:

Two major impacts have resulted from this study so far. (1) During times of severe drought, there are no safe areas to plant disease-free seed except the most upriver areas that can be tolerated. (2) SPF juvenile oysters placed at sites remote from natural oyster populations harboring *P. marinus* acquire infections at similar rates as SFP juveniles placed adjacent to natural populations. This finding could have a major impact on planting of both natural and hatchery seed, which some have thought would remain disease free if planted away from existing populations or on natural bars that had been cleaned of older infected oysters.

PROJECT PUBLICATIONS:

George R. Abbe, Brian W. Albright, Carol B. McCollough, Christopher F. Dungan and Stephen J. Jordan. 2002. Environmental effects on *Perkinsus marinus* infection rates, growth and survival among dermo-disease-free juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland. Journal of Shellfish Research 21:371. (This was also an invited paper presented at the National Shellfisheries Association meeting in Mystic, Connecticut in April 2002.)

Carol B. McCollough, Christopher F. Dungan, Stephen J. Jordan, George R. Abbe and Brian W. Albright. 2002. *Perkinsus marinus* infection rates in specific-pathogen-free juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland. Journal of Shellfish Research 21:375. (An invited paper presented at the National Shellfisheries Association meeting in Mystic, Connecticut in April 2002.)

FUNDING PERIOD: Jan. 1, 2000 – Dec. 31, 2001

PROJECT TITLE: Environmental effects on *Perkinsus marinus* infection rates, growth and survival among dermo-disease-free juvenile oysters planted at three salinity regimes in an enzootic Chesapeake Bay Oyster Recovery Area

PRINCIPAL INVESTIGATOR: George Abbe
AFFILIATION: Academy of Natural Sciences Estuarine Research Center
CO-INVESTIGATORS and AFFILIATIONS: Chris Dungan and Steve Jordan, Sarbanes Cooperative Oxford Laboratory

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PROJECT RESULTS:
Specific pathogen-free (SPF) oysters were set on oyster shell and transplanted to three sites on natural oyster bars in the Patuxent River, Maryland along a salinity gradient to investigate environmental effects of *Perkinsus marinus* on infection rates, growth and survival. Spat were deployed at each site for disease monitoring, and 100 were followed for growth and mortality. From September 2000 to September 2001salinities at Holland Point (HP, upper river), Gatton (GAT, mid) and Town Creek (TC, lower) averaged 11.1, 13.0 and 14.4, respectively. Oysters were examined monthly for growth and mortality and 30 were collected from each site for assay of *P. marinus* infections by the whole body burden technique. This allowed determination of time to initial infection and subsequent progression of dermo disease reflected by parasite burdens. An additional 30 from the natural population at each site were also examined monthly by rectal tissue assay.

Oysters (initially 25 mm) at HP, GAT and TC grew 23, 34 and 27 mm, respectively, and survival was 95, 98 and 94% during the first 12 months. Three discrete spat sets and deployments were made: September 2000, June 2001, and August 2001. Animals at sites TC and GA September 2000 deployments acquired *P. marinus* infections within 2 weeks, with prevalences of 10% and 3% respectively. Positive results at low prevalences and intensities continued for 8 weeks. Sites TC and GA June 2001 deployment acquired infections within 2 weeks, with 10% and 13% prevalences respectively, and positive results continued for 8 weeks with increasing prevalences. Site HP acquired one infection (3%) between 2 and 4 weeks. Positive results continued through two additional 4-week sampling intervals, with increasing prevalences. All August 2001 deployments acquired infections within 2 weeks, with prevalences of 7%, 87%, and 3% respectively, and positive results continued for 8 weeks. Infection intensities among these samples ranged from 1-23 hypnospores per host animal. The GA site was 100% infected by 8 weeks post-deployment. These results show that juvenile oysters acquire *Perkinsus marinus* infections as early as 2 weeks after placement in dermo-disease endemic areas, and that these infections persist in the planted populations over time.

Mean intensity of dermo disease among feral populations (on a scale of 0-7) at HP, GAT and TC ranged from 1.1 to 4.2, 0.7 to 4.6 and 0.7 to 4.7, respectively, and averaged 2.51, 2.72 and 2.79. It appears that salinity, varying within the range of our sites, had little effect on growth, survival or infection intensity during the first year.

IMPACTS and/or BENEFITS:
There was little impact evidenced from the first year of this study other than development of our overall strategy and techniques that was to carry over into year 2 and 3. Preliminary data suggested that a mid-river site might be the best area to locate oysters, but additional data during subsequent years dictated otherwise.

PROJECT PUBLICATIONS:

George R. Abbe, Brian W. Albright, Carol B. McCollough, Christopher F. Dungan and Stephen J. Jordan. 2002. Environmental effects on *Perkinsus marinus* infection rates, growth and survival among dermo-disease-free

juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland. Journal of Shellfish Research 21:371. (This was also an invited paper presented at the National Shellfisheries Association meeting in Mystic, Connecticut in April 2002.)

Carol B. McCollough, Christopher F. Dungan, Stephen J. Jordan, George R. Abbe and Brian W. Albright. 2002. *Perkinsus marinus* infection rates in specific-pathogen-free juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland. Journal of Shellfish Research 21:375. (An invited paper presented at the National Shellfisheries Association meeting in Mystic, Connecticut in April 2002.)

FUNDING PERIOD: 10/1/01-9/31/03

PROJECT TITLE: In Situ Determination of *Perkinsus marinus* Transmission Dynamics in Low Salinity Habitats: Implications for Disease Avoidance Management Strategies and Oyster Restoration

PRINCIPAL INVESTIGATOR: Kennedy T. Paynter
AFFILIATION: Department of Biology, UMCP
CO-INVESTIGATORS and AFFILIATIONS: Eugene Burreson, Kimberly Reece, Lisa Ragone-Calvo. Virginia Institute of Marine Science

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PROJECT RESULTS:

Elucidation of *P. marinus* transmission dynamics requires examination of environmental parasite abundances and in situ transmission rates. With the recent advancement of molecular techniques for the detection of *P. marinus* and the acquisition by VIMS of a state-of-the-art real-time PCR system for DNA quantification, we have the required tools for sensitive, specific, and quantitative detection of *P. marinus*. We have conducted an investigation funded by ODRP, in which these molecular tools have been optimized and utilized to quantify the number of parasite cells in water samples collected at several sites within low and moderate salinity areas in both Maryland and Virginia. Concurrently we monitored infection acquisition by naïve sentinel oysters deployed at the same sites, and oyster mortality and *P. marinus* prevalence in local native oyster populations.

During this current funding cycle we have developed and refined our monitoring protocols and the PCR assay. This experimental framework has successfully been established and our results to date are beginning to demonstrate linkages between infected host mortality, water column parasite abundance, and infection transmission to naïve sentinel oysters in the James River. Although much of the data from Maryland has yet to be analyzed we anticipate lower parasite abundances since salinity at the Maryland sites are lower. As with any *in situ* investigation conducted in a complex system, the interannual variation of a diversity of environmental parameters can affect the strength of correlations between dependent variables and may lead to erroneous interpretations of the results. For this reason, validation of the results of the two-year study, currently in progress, will require additional years of study, especially given the nearly two year old drought the Chesapeake Bay watershed is currently experiencing. We are currently proposing to extend our present study an additional two years and to expand both temporally and spatially our current sampling regime.

IMPACTS and/or BENEFITS:

This research will contribute data important to our understanding of disease processes in the Chesapeake Bay, allowing managers to better control and manage disease problems plaguing the oyster industries of both Virginia and Maryland. Results from this research could lead directly to changes in management practices that could significantly reduce disease problems in certain areas. It will also provide data critical for oyster population/disease models being developed for oyster populations in the Chesapeake Bay.

PROJECT PUBLICATIONS:

In situ determination of *Perkinsus marinus* transmission dynamics. Lisa Ragone Calvo, Corinne Audemard, Kimberly Reece, Eugene Burreson, Virginia Institute of Marine Science; and Kennedy Paynter, University of Maryland. Presented at the International Conference on Shellfish Restoration, Charleston, SC.

FUNDING PERIOD: 9/1/98 to 8/31/00

PROJECT TITLE: Predicting time to critical levels of *Perkinsus marinus* in eastern oysters, *Crassostrea virginica* : a new tool for increasing oyster production.

PRINCIPAL INVESTIGATOR: Sammy M. Ray
AFFILIATION: Dept.of Marine Biology, Texas A&M University, Galveston, TX 77553

CO-INVESTIGATORS and AFFILIATIONS: Thomas M. Soniat, Department of Biology, Nicholls State University Thibodaux, LA 70310; Enrique Kortright, Kortright Corporation, 102 Allendale Dr., Thibodaux, LA 70301

PROJECT RESULTS:
In the initial funding cycle the DermoWatch project was established as an online community for the management of *Perkinsus marinus* in oysters. The project phases included sampling oysters and disease analysis, the establishment of a web site (www.blueblee.com/dermo), and modeling for the calculation of a time to a critical level of disease. Inputs into the model were established as initial weighted incidence of disease, oyster length, and water temperature and salinity.

The home page of the web site provides the most recent data for the Bay. Clicking on a station on a map of Galveston Bay gives a history of all of the data collected from the location. Three leases and four public reefs are sampled monthly, and levels of *Perkinsus* (*=Dermocystidium*) *marinus* are determined. A DermoWatch calculator was developed which allows anyone with information on initial weighted incidence of disease, oyster length, and water temperature and salinity to calculate a time to critical level of disease. Thus the web site is useful to users throughout the range of Dermo – from Maine to Mexico.

IMPACTS AND BENEFITS:
DermoWatch is a web-based community of scientists, managers and oyster growers. It provides recent and historical data on the occurrence and progression of Dermo disease in Galveston Bay, Texas. The web site calculates a time to a critical level of disease. This is an estimate of the time that it would take the parasite to reach a critical level, assuming no change in temperature and salinity. Thus, growers and managers have a record of the present status of disease, a history of past conditions and a prediction of the future direction of disease progression.

With DermoWatch *Perkinsus* ceases to be an unseen killer, and growers can follow disease conditions as they relate to the mortalities they observe. The estimate of a time to critical level of disease allows an oyster grower to make an informed decision to harvest immediately, move oysters to a lower salinity area, or keep the crop in place.

PROJECT PUBLICATIONS:

The first publications from the project were in 2002 and appear in the report for the second funding cycle.

FUNDING PERIOD: 9/1/99 to 10/8/03 (continuation)

PROJECT TITLE: DermoWatch: a web-based approach for managing Perkinsus marinus disease of oysters.

PRINCIPAL INVESTIGATOR: Sammy M. Ray
AFFILIATION: Department of Marine Biology, Texas A&M University, Galveston, TX 77553
CO-INVESTIGATORS and AFFILIATIONS: Thomas M. Soniat, Department of Biology, Nicholls State University, Thibodaux, LA 70310; Enrique Kortright, Kortright Corporation, 102 Allendale Dr., Thibodaux, LA 70301

PROJECT RESULTS:
Progress is continually being made on all phases of the project. The project phases include sampling oysters and disease analysis, web site improvement, and constructing a continuous monitoring station. The core sampling site is in Galveston Bay, where three leases and four public reefs are sampled monthly, and levels of *Perkinsus* (=*Dermocystidium*) *marinus* are determined. The field sampling and oyster analysis is kept recent, and can be seen from the home page.

New oyster sampling and disease analysis has been conducted from new locations, including the state public grounds in Louisiana and at the site of a new environmental-monitoring station in Bay Tambour near Cocodrie, Louisiana. The Louisiana Department of Wildlife and Fisheries has collected the oyster samples and the disease assays have been completed. The new Louisiana public areas (and sites) are Lake Calcasieu (West Cove, East Side), Vermillion Bay (Bayou Blanc), Sister Lake (North Bay Junop, South Bay Junop, Old Camp, Grand Pass), Barataria Bay (Hackberry Bay), Breton Sound (North Black Bay, South Black Bay, Mozambique Point, Bay Crabe, Lonesome Island, Bay Gardene, Telegraph Point), and Mississippi Sound (Three Mile Point, Cabbage Reef). The public ground sites are sampled annually, whereas the Bay Tambour site is sampled monthly. The data will be available online when a new web site is completed.

A new web site (www.dermowatch.org), with a new look incorporating new stations, is nearing completion. The new home page includes a map of all sampled areas. Clicking on an area will give a list of sites with access to recent and historical data and a finer-scale map. The expansion of the DermoWatch program to include more sites and more participants has prompted us to develop a new data entry process. The new process is more secure and flexible since it includes individualized entry and data form generation.

The modeling to link continuous data from the monitoring station to the web site is largely complete, since it varies little from the buoy-data utility already in place (www.blueblee.com). The modeling to produce graphs of water temperature, salinity and a time-to-critical level of disease is completed; the new web page will give a time-course prediction of weighted incidence of disease that changes with the continuously-monitored values of temperature and salinity.

A station to monitor environmental conditions at the Bay Tambour has been competed. The parameters that are being measured include wind speed and direction, solar radiation, rainfall, barometric pressure, dissolved oxygen, fluorescence (chlorophyll), turbidity, and water temperature and salinity. The station is collecting data to a data logger, but the data are not yet being transmitted to the marine lab at Cocodrie. Completion of the transmission phase has been delayed by resolvable complications due to competing transmission signals from the Bay Saint Elaine Oil Field and by delays due to tropical storm Isadore and hurricane Lilli.

IMPACTS AND BENEFITS: DermoWatch is a web-based community of scientists, managers and oyster growers. It provides recent and historical data on the occurrence and progression of Dermo disease in Texas and Louisiana. The web site calculates a time to a critical level of disease.

This is an estimate of the time that it would take the parasite to reach a critical level, assuming no change in temperature and salinity. Thus, growers and managers have a record of the present status of disease, a history of past conditions and a prediction of the future direction of disease progression.

With DermoWatch *Perkinsus* ceases to be an unseen killer, and growers can follow disease conditions as they relate to the mortalities they observe. The estimate of a time to critical level of disease allows an oyster grower to make an informed decision to harvest immediately, move oysters to a lower salinity area, or keep the crop in place.

PROJECT PUBLICATIONS:

Kortright, E.V., T.M. Soniat, and S.M. Ray. 2002. Web model estimates oyster parasite time-to-critical levels. Sea Technol. 43(4):43-46

Ray, S.M., T.M. Soniat, E.V. Kortright, and L. Robinson. 2002. Recent trends in levels of infection of *Perkinsus marinus* in oysters from Galveston Bay, Texas: results of the DermoWatch monitoring program. J. Shellfish Res. 21:375.

Soniat, T.M., E.V. Kortright, and S.M. Ray. 2002. DermoWatch: a web-based approach for monitoring the oyster parasite *Perkinsus marinus* (*Dermocystidium marinum*) J. Shellfish Res. 21:389.

FUNDING PERIOD: 10/01/97-09/31/99

PROJECT TITLE: Development and Management Applications of a Dual-Disease (MSX and Dermo) Model for Chesapeake Bay Oyster Populations

PRINCIPAL INVESTIGATOR: Eileen E. Hofmann
AFFILIATION: Old Dominion University

CO-INVESTIGATORS and AFFILIATIONS: J.M. Klinck, Old Dominion University; E.N. Powell and S.E. Ford, Haskin Shellfish Research Laboratory, Rutgers University; S.J. Jordan, Paul S. Sarbanes Cooperative Oxford Laboratory

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PROJECT RESULTS:
This project was a joint research effort between E. Hofmann and J. Klinck at Old Dominion University; E. Powell and S. Ford at the Haskin Research Laboratory, Rutgers University; and S. Jordan at the Oxford Cooperative Laboratory in Oxford, MD. This final report includes activities for the Old Dominion University component of the project. However, because of the cooperative nature of this study, the efforts of other PIs are also reflected in this report.

The primary objectives of this research are to: 1) use the *H. nelsoni* model to investigate the influence of biological processes and environmental variability on MSX disease prevalence and infection intensity in Eastern oyster, *Crassostrea virginica,* populations, and 2) understand the effect of long-term climate variations on the prevalence and intensity of MSX disease.

This project has resulted in the development of an oyster-*H. nelsoni* model that is physiologically-based and is structured around the transmission, proliferation and death rates of the parasite. Environmental conditions of temperature, salinity and oyster food supply provide the external forcing that results in variations in these biological rates. This model has been used to investigate the role of environmental variations versus biological processes in controlling the prevalence and intensity of MSX disease in oyster populations. The oyster-*H. nelsoni* model is capable of accurately simulating epizootics of MSX disease that occurred in Delaware Bay in the 1960s and those that occurred in Chesapeake Bay during the 1990s. Our research with the oyster-*H. nelsoni* model has also focused on developing simulations that will allow investigation of the effect of long-term climate variations on MSX disease prevalence and intensity in oyster populations in Delaware and Chesapeake Bay. The area of research has numerous implications for management of oyster populations.

Throughout this project, we have made several presentations on the oyster-*H. nelsoni* model at national and international scientific meetings in order to make the broader community aware of our work. These are listed below, as are the corresponding abstract publications. The oyster- *H. nelsoni* model and results obtained with this model are described in three manuscripts that are in press in *Journal of Shellfish Research,* one paper published in *Science* and one paper that is submitted for a special edition of *Hydrobiologia* on diseases in the ocean. These are also listed below.

Our research on using models to understand environmental and biological interactions that produce observed prevalences and intensities in oyster populations was recognized nationally by an invitation for us to participate in a special symposium on "Diseases of the Ocean: A New Environmental Challenge" that was convened as part of the 1999 Annual Meeting of the American Association for the Advancement of Science (AAAS). This symposium was designed to increase awareness of the importance of disease in regulating marine populations.

Our research was further recognized by an invitation to participate in a panel discussion that was broadcast from the AAAS meeting as part of National Public Radio's Science Friday program that is hosted by Ira Flatow. The

topic of the Science Friday broadcast was "Diseases in the Ocean" and E. Hofmann participated in the panel discussion as the representative of this project. Subsequently, E. Hofmann contributed to the Ocean Report, which is an outreach effort sponsored by the Pew Charitable Trusts that is broadcast daily on more than 200 radio stations. The program, which is hosted by Peter Benchley, was broadcast on 22 June 1999 and was entitled "Oyster Parasites".

E. Hofmann was invited to visit Southampton University, Southampton, NY to participate in a lecture series that is designed to make undergraduates aware of current research topics in marine science. The oyster-*H. nelsoni* model was the topic of the lecture that was presented, which was attended by about 60 students.

Recently the National Research Council's Committee on Climate, Ecosystems, Infectious Disease, and Human Health convened a workshop that was focused on interactions between environmental effects and diseases. We were invited to participate in this workshop and to make a presentation on our oyster disease models. We were also asked to provide input for the development of future studies to be undertaken by the NRC on disease and environmental and long-term climate interactions.

PROJECT PUBLICATIONS:

Ford, S.E., E.N. Powell, J.M. Klinck, E.E. Hofmann, Modeling the MSX Parasite in Eastern Oyster (*Crassostrea virginica*) Populations. I. Model Development, Implementation and Verification, *Journal of Shellfish Research,* 18, 475-500, 1999.

Harvell, D.C., K. Kim, J.M. Burkholder, R.R. Colwell, P.R. Epstein, J. Grimes, E.E. Hofmann, E. Lipp, A.D.M.E. Osterhaus, R. Overstreet, J.W. Porter, G.W. Smith and G. Vasta, Diseases in the Ocean: Emerging Pathogens, Climate Links, and Anthropogenic Factors, *Science*, 285, 1505-1510.

Hofmann, E.E., S.E. Ford, E. N. Powell and J.M. Klinck Modeling Studies of the Effect of Climate Change on MSX Disease in Eastern Oyster (*Crassostrea virginica*) Populations, *Hydrobiologia,* 460, 195-212, 2001.

Paraso, M., Ford, S.E., E.N. Powell, E.E. Hofmann, J.M. Klinck, Modeling the MSX Parasite in Eastern Oyster (*Crassostrea virginica*) Populations. II. Salinity Effects, *Journal of Shellfish Research*, 18, 501-516, 1999.

Powell, E.N., J.M. Klinck, S.E. Ford, E.E. Hofmann, S.J. Jordan, Modeling the MSX Parasite in Eastern Oyster (*Crassostrea virginica*) Populations. III. Regional Application and the Problem of Transmission, *Journal of Shellfish Research*, 18, 517-537, 1999.

PROJECT NUMBER: VGMSC Sub-Contract # 5-29457
FUNDING PERIOD: 10/01/99-07/31/02

PROJECT TITLE: Modeling the Effects of Climate Variability on the Prevalence and Intensity of Dermo and MSX Diseases in Eastern Oyster Populations

PRINCIPAL INVESTIGATOR: Eileen E. Hofmann
AFFILIATION: Old Dominion University
CO-INVESTIGATORS and AFFILIATIONS: J.M. Klinck, Old Dominion University; S. Ford and E. Powell, Rutgers University; S. Jordan, Sarbanes Cooperative Oxford Laboratory; E. Burreson, Virginia Institute of Marine Science

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PROJECT RESULTS:
The specific research topics investigated as part of this study are: 1) the effect of climate variability on initiating and controlling epizootics of Dermo and MSX disease in oyster populations in Chesapeake and Delaware Bays; 2) the interaction of Dermo and MSX disease in Chesapeake and Delaware Bays oyster populations; 3) the factors (environmental and biological) that account for differences in prevalence and intensity in the two diseases over a latitudinal gradient within Chesapeake Bay and between Chesapeake and Delaware Bays; and 4) the value of nowcasts of oyster disease prevalence and intensity in Chesapeake Bay.

The first three objectives were addressed with a series of simulations that used output of the Hadley Center Climate Model to specify the predicted change in salinity that would be encountered in Chesapeake Bay for conditions of continued climate warming resulting from a doubling of CO_2. Additional simulations considered the effect of variability in food supply and environmental conditions, such as periods of high food years followed by low food years or wet years followed by periods of dry years. The results of these simulations were compared to simulations that used mean conditions that were established using time series of temperature, salinity, chlorophyll and total suspended solids from the Chesapeake Bay EPA data set for the period 1987 to 1998 for several sites in Chesapeake Bay. The simulations were done using the Dermo and MSX disease models that are coupled to the oyster population dynamics model. The results of these simulations are now being prepared for publication.

The fourth objective was addressed with a model that was developed for the management of fished oyster populations in which disease mortality is a controlling influence. The model requires a quantitative estimate of abundance by size class, some knowledge about growth rates to establish the size range recruiting into the fisheries, and an estimate of the anticipated natural mortality rate. The model permits investigation of scenarios that include a range of allocations, timing of fishing seasons, variation in fishing efforts within seasons to establish a preferred harvest level, variation in the distribution of fishing among beds to minimize over-harvesting of disease-affected beds (area management), and rebuilding plans to increase total stock abundance after epizootic mortality or periods of over-harvesting. Another management-oriented model has been developed and calibrated using long-term oyster monitoring data from Maryland's Chesapeake Bay. Means and standard deviations of natural mortality (principally from diseases), fishing mortality and recruitment were derived from a 17-year time series (1985-2001). Population parameters can be adjusted to simulate the effects of management options on the long-term abundance of the market oyster stock.

The simulations show that conditions of doubled CO_2, which results in increased precipitation in the Chesapeake Bay region and thus lower salinity, will produce a decrease in the prevalence and intensity of Dermo and MSX disease in oyster populations. Continued conditions of decreased salinity results from the high precipitation (e.g., more wet years) will further reduce prevalence and intensity of the two diseases. However, for reduced salinity conditions, there is a trade-off between reduced Dermo and MSX disease prevalence and intensity and reduced reproductive capacity of the oysters. Extensive periods of low salinity significantly reduce the reproductive output of the oysters and as a result recruitment to the population is reduced. However, additional simulations show that conditions of increased food can offset/overwhelm effects of either increased or decreased salinity on oyster

reproduction. These results show clearly the need to characterize the food environment of the oyster populations in Chesapeake Bay. Another factor for further investigation is the effect of warming temperatures on Dermo and MSX disease and oyster reproduction.

The total oyster biomass changes calculated from the climate change simulations suggest that the northern Chesapeake Bay oyster populations may not be viable over the long term without external inputs of juveniles. The simulations using conditions characteristic of the Rappahannock and York Rivers show that oyster populations at these sites will either increase or have stable biomass over time for most of the climate change conditions tested with model. The implication of these simulations is that the southern Chesapeake Bay oyster populations may sustain the Bay-wide oyster fishery during periods of climate change that result in a decrease of Bay salinity. This is an important result for development of long-term management strategies for oyster populations.

Simulations with the fisheries model show that appropriate timing of the fishing season with respect to the timing of disease mortality can more than double the yearly allocation to the fishery. Besides disease, the other model parameter that most affects the simulation outcomes is the abundance of submarket-size oysters that can be expected to recruit to the fishery in the simulated year. Population stability is strongly determined by the number of recruits available to replace the deaths that decimate the market-size population each year. The model points to the critical need to understand population dynamics and survival of size classes below market size that are not often the targets of investigation. Simulations with the Maryland model also have highlighted the importance of recruitment and the need to develop an age-structured model that includes a growth component, as well as a better understanding of the stock-recruitment relationship. Baseline simulations indicate that the average rates of disease mortality, fishing mortality and recruitment that have been observed in Maryland since 1985 are not sustainable. The market oyster stock will continue an exponential decline unless mortality is reduced, recruitment increased, or both. Reducing fishing mortality from F=0.6 to F=0.4, or doubling of recent hatchery-based stock enhancement efforts could reverse the downward trend over the next decade.

We undertook a new modeling approach that is based on basic metabolic processes, as part of our continuing effort of development of models for shellfish populations. This consisted of development of a biochemically-based model for simulating the growth, development, and metamorphosis of larvae of the Pacific oyster, *Crassostrea gigas*. This model, which is the first of its type, defines larvae in terms of their gross biochemical composition: protein, neutral lipid, polar lipid, carbohydrate, and ash content. The model includes parameterizations for larval filtration, ingestion, and respiration, which determine growth rate, and processes controlling larval mortality and metamorphosis. The initial biochemical content of the larva is determined by the composition of the egg. Changes in the initial ratios of protein, carbohydrate, neutral lipid and polar lipid occur as the larva grows and in response to the biochemical composition of available food.

The larval growth model was developed for a single individual. Thus, we also developed an approach for extending the model results to an entire population through use of probability distributions that are applied to metabolic processes, such as respiration or assimilation. This approach allows genetic variability within a population to a factor in determining larval survival.

IMPACTS and/or BENEFITS:
The results of the simulation models from this effort show the importance of including climate change effects in development of long-term management strategies for oyster populations in Chesapeake Bay.
The biochemically-based model developed for *C. gigas* larvae provides a mechanistic structure for models of shellfish populations. This approach is already being incorporated into the development of models for other shellfish, such as the hard clam and the butter clam.

The fisheries management models provide quantitative tools for setting harvesting times, quotas and evaluating

other management options for diseased oyster fisheries. These models are now being applied to oyster populations
in Delaware and Chesapeake Bays.
The primary benefit of this research is the development and availability of models for oyster-disease interactions and a fisheries model for managing an oyster fishery that is impacted by disease. These models are potentially of use to managers. The fisheries model is now being used to guide management decisions for oyster populations in Chesapeake and Delaware Bays.

PROJECT PUBLICATIONS:

Bochenek, E.A., J.M. Klinck, E.N. Powell, E.E. Hofmann, A biochemically based model for the growth and development of *Crassostrea gigas* larvae, *Journal of Shellfish Research*, 20, 243-265, 2001.

Hofmann, E.E., S.E. Ford, E.N. Powell, J.M. Klinck, Modeling studies of the effect of climate change on MSX disease in eastern Oyster (*Crassostrea virginica*) populations, In: *The Ecology and Etiology of Newly Emerging Marine Diseases*, J.W. Porter, ed., *Hydrobiologia*, 460, 195-212, 2001.

Hofmann, E.E., J.M. Klinck, E.N. Powell, S.E. Ford, S. Jordan, E. Burreson, Modeling studies of climate variability and disease interactions in eastern oyster populations, manuscript in preparation.

Hofmann, E.E., E.N. Powell, E.A. Bochenek, J.M. Klinck, Critical conditions for larval success: influence of environmental food supply on survival of *Crassostrea gigas* larvae: a modeling study, *Marine Ecology Progress Series*, submitted.

Jordan, S.J., K.N. Greenhawk, C.B. McCollough, J. Vanisko, M.L. Homer, Oyster biomass and abundance in northern Chesapeake Bay: trends and forecasts, *Journal of Shellfish Research*, submitted.

Klinck, J.M., E.N. Powell, J.N. Kraeuter, S.E. Ford, K.A. Ashton-Alcox, A fisheries model for managing the oyster fishery during times of disease, *Journal of Shellfish Research*, 20, 977-989, 2001.

Powell, E.N., E.A. Bochenek, J.M. Klinck, E.E. Hofmann, Influence of food quality and quantity on the growth and development of *Crassostrea gigas* larvae: a modeling study, *Aquaculture*, 210, 89-117, 2002.

Powell, E.N., E.A. Bochenek, J.M. Klinck, E.E. Hofmann, Critical conditions for larval success: influence of short-term variations in food supply on survival of *Crassostrea gigas* larvae: a modeling study, *Marine Ecology Progress Series*, submitted.

FUNDING PERIOD: 1999

PROJECT TITLE: Oyster Predator-Prey Interactions: Roles of Different Predators, Seasonality, Spatial Variation and Deterrents.

PRINCIPAL INVESTIGATOR: Kenneth Brown
AFFILIATION: Department of Biological Sciences, Louisiana State University
CO-INVESTIGATORS AND AFFILIATIONS: Gary Peterson[2], Mike McDonough[1], and Charles Ramcharan[1]. [1] Department of Biological Sciences, [2] Coastal Ecology Institute. Louisiana State University

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PROJECT RESULTS:

We conducted both small-scale laboratory and large-scale field experiments to study the roles of scent deterrents, snail versus fish predators, estuarine versus coastal conditions, and seasonality in explaining mortality rates of oysters (*Crassostrea virginica*). Preliminary laboratory experiments at the Grand Terre Marine Laboratory indicated that small, single oysters, such as those planted on oyster leases, are extremely vulnerable to predation by black drum (*Pogonias cromis*). Experiments in 20,000 L raceways indicated the scent of dead conspecific drum reduced mortality rates from feeding fish by 46%, but variation among experimental trials was so large that the effect was not significant. In large-scale field experiments at both estuarine and coastal locations in Barataria Bay, Louisiana, we tested whether the scent of dead conspecifics would lower mortality rates to fish in separate experiments in the fall and spring. Percent survival of oysters varied from 2 to 66%, and tended to be highest in the fall at the estuarine site. Percent mortality caused by fish varied from 61 - 99%, and was highest in the spring at the coastal site. Percent mortality caused by oyster drills (*Stramonita haemastoma*) varied from 1 - 38% and was highest in the fall at the estuarine site. Scent significantly reduced fish feeding rates only at one of the sites, and then only for one of four weeks in the fall experiment. We conclude that oyster drills and black drum pose serious predation risks for oysters, both when seed oysters are planted in the fall, and before oysters are harvested in the spring. However, the scent of dead conspecifics, considered a deterrent by local lease holders, does not seem effective in reducing mortality rates caused by fish. We are currently investigating whether acoustic cues are effective deterrents for fish predation.

PROJECT PUBLICATIONS

Brown, K.M., C. Ramcharan, B. Lezina, G. Peterson, and P. Banks. 2001. Novel deterrents to black drum predation on oyster leases. World Aquaculture Society Meeting at Lake Buena Visata, FL, p. 91 (abstract).

Kenneth M. Brown, [1]* Gary Peterson[2], Mike McDonough[1], and Charles Ramcharan[1]. [1] Department of Biological Sciences, [2] Coastal Ecology Institute. Louisiana State University. Oyster predator-prey interactions: roles of different predators, seasonality, spatial variation and deterrents. 31st Marine Benthic Ecology Meeting, 21-24 March 2002, Orlando, FL. Hosted by the Florida State University.

FUNDING PERIOD: 2001-03 (on-going)

PROJECT TITLE: Oyster Harvest Efforts in Pre-Diversion Barataria Bay During a Decade of Salinity Shifts, 1990-99

PRINCIPAL INVESTIGATOR: Earl J. Melancon, Jr.
AFFILIATION: Biology Department, Nicholls State University, Thibodaux, LA 70310
CO-INVESTIGATORS and AFFILIATIONS: Eric Swenson, Louisiana State University; Peter Vujnovich, Jr., Captain Pete's Oyster Company, Port Sulfur, Louisiana.

PROJECT RESULTS TO DATE (on-going)

We have delineated four oyster microhabitat areas in Barataria Bay based on summarizing commercial harvest records from an oyster vessel over the decade, 1990-99. As the bay's salinity habitat shifted during wet and dry climate periods, the oysterman correspondingly shifted his fishing efforts up and down bay to where oysters had set and survived to commercial size, generally ^{3}65mm in shell length. The microhabitat areas are being referenced against the 1990-99 semi-annual wet-dry climatic classification index for the bay system as developed by Swenson and Turner (1998); Detailed methods can be found in their publication. Semi-annual mean isohalines in 5-ppt increments are also being generated for the bay to correlate with fishing effort and the climatic classification index. We have also collected monthly oyster dredge samples during the period summer to fall 2002 to profile the oyster populations within each area and to correlate with prevailing salinity conditions in the bay; Oyster parameters measured were population size frequency distribution, condition index, gonad index, and sex ratio.

Area 1 is the most down-estuary, and highest salinity site, and preliminary results indicate a good correlation with wet-year estuary conditions which are conducive to oyster survival. Area 2 is adjacent to and up-estuary from area 1, and corresponds to habitat where some oysters survived regardless of wet or dry estuary conditions. Area 3 also has oysters to survive in wet-dry conditions, but is more up-estuary than Area 2. Area 4 is far up-estuary and conducive to oyster spat settlement and survival when salinities increase due to drought or near-drought conditions. Area productivity generally lagged one to three years behind salinity shifts that allowed for oysters to set, survive and grow to commercial size. The research is on going.

IMPACTS and/or BENEFITS:
The primary goal is to relate the pre-diversion natural shifts in commercial utilization of oyster leases within Barataria Bay to post-diversion shifts in salinity habitat. The ultimate goal is to investigate how habitat shifts affect an oysterman's ability to make a living and how wise utilization of diversions, within the context of wetlands management, may reduce negative fishery impacts.

PROJECT PUBLICATIONS:
No referred journal publications have yet been generated by this research.

FUNDING PERIOD: 9/1/97 to 8/31/99

PROJECT TITLE: Video Documentary on the Rise and Fall of the Chesapeake Oyster Fishery

PRINCIPAL INVESTIGATOR: Michael W. Fincham
AFFILIATION: Maryland Sea Grant
CO-INVESTIGATORS and AFFILIATIONS:
John R. Greer, Maryland Sea Grant

PROJECT RESULTS:
This project allowed us to complete several videos on oyster restoration in the Chesapeake and lay the foundation for a long-form documentary on the rise and fall and restoration of the Chesapeake oyster fishery. The major results include

- Two versions of the video, *From the Bottom Up: Restoring Oyster Reefs in Chesapeake Bay.*

- Research into the photographic archives, including the Library of Congress, the National Archives, the Maryland State Archives, CBL, Enoch Pratt Library, Chesapeake Bay Maritime Museum, Calvert Marine Museum, Tawes Museum, Baltimore Museum of Industry, Maryland Historical Society, National Geographic, Virginia Institute of Marine Science and some independent sources. As a result we now have identified several hundred photocopied images, most of them historic, of oyster harvesting, boats, oyster houses, oyster-related industry (shucking, canning, fertilizer plants, etc.), rural and urban (Baltimore, Washington, Annapolis) harbors, pierside activities, wholesale and retail markets, portraits, and communities.

- Research into film archives included many of the institutions listed above as well as additional sources. We have found some of the earliest footage of the industry, including silent and early sound film of harvesting and shucking as well as a never-seen interview with Reginald Truitt.

- Field shoots in Maryland and Virginia on the oyster industry and science research. Industry shoots include sequences and interviews on oyster dredging, tonging, planting, shucking and boat building. Video shoots on oyster research include field and laboratory sequences and interviews focused on MSX, hatcheries, nurseries, and reef restoration.

- Funding research and partnerships are currently being explored with a number of foundations and with public television for completing a long-form television documentary and a series for educational use.

IMPACTS and/or BENEFITS:
The completed video versions *of From the Bottom Up: Restoring Oyster Reefs in Chesapeake Bay,* have been used to educate key audiences about the science behind the growing effort at oyster restoration. Those audiences include legislators, both federal and state, research meetings, oyster industry events, school classes, maritime musems, and the annual meeting of the Society of Environmental Journalists.

PROJECT PUBLICATIONS:
Two video versions (11 minutes and 14 minutes): *From the Bottom Up: Restoring Oyster Reefs in Chesapeake Bay*

WORKGROUP 2

GENETICS AND OYSTER POPULATIONS

NOTES

WORKGROUP 2

GENETICS AND OYSTER POPULATIONS

Development of Nuclear DNA markers and pedigreed families for disease resistance and genetic mapping in the eastern oyster. Patrick M. Gaffney, Standish K. Allen, Jr. and James C. Pierce, 1995.

Genetic monitoring of tetraploid hybrid oysters. Patrick M. Gaffney, Ximing Guo, 1995.

Evaluation of American oyster stocks for resource rehabilitation disease resistance and genetics. Patrick M. Gaffney, K. Paynter and D. Meritt, 1995-1996.

Physiological and genetic correlates of Dermo disease Resistance in the Eastern oyster: An interdisciplinary approach. Patrick Gaffney, Steven Kaattari and Mohammed Faisal, 1997-1998

Development of a moderate density linkage map of the eastern oyster Crassostrea virginica: Identification of disease resistant genes. Patrick Gaffney and Kimberly Reece, 2000.

Breeding and evaluation of Oyster (*Crassostrea virginica*) strains selected for resistance to MSX, Dermo and JOD. Ximing Guo, Susan Ford, Gregory DeBrosse and Roxanna Smolowitz, Dec. 1, 1999-Sept 30, 2001

Breeding, evaluation and molecular analysis of oyster strains selected for resistance to MSX Dermo and JOD. Ximing Guo, Susan Ford, Gregory DeBrosse and Roxanna Smolowitz, Oct. 1, 2001-Sept 30, 2003

Combined use of a lytic peptide and protease inhibitors: A novel approach to eliminate *Perkinsus marinus* in eastern oysters. Jerome F. La Peyre, Richard K. Cooper and Terrence R. Tiersch, September 1, 1997-August 31, 1999.

Optimization of gene delivery for improved oyster health. Richard K. Cooper, Terrence R. Tiersch and Jerome F. La Peyre, October 1, 1999-September 30, 2001.

Accelerated selective breeding to develop Dermo resistance to oysters in the Gulf of Mexico and identification of potential mechanisms for increased disease resistance. Jerome F. La Peyre and John E. Supan, October 1, 2001-September 31, 2003.

Creation of an oyster cell line in *C. virginica*. Jane C. Burns and Carolyn Friedman.

FUNDING PERIOD: 1995

PROJECT TITLE: Development of Nuclear DNA Markers and Pedigreed Families for Disease Resistance and Genetic Mapping in the Eastern Oyster

PRINCIPAL INVESTIGATOR: Patrick M. Gaffney
AFFILIATION: University of Delaware
CO-INVESTIGATORS and AFFILIATIONS: Standish K. Allen, Rutgers University; James C. Pierce, Philadelphia College of Pharmacy and Science

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PROJECT RESULTS:
The bacteriophage P1 cloning system was used to construct a high-quality, high molecular weight genomic library. This is the first high molecular weight genomic library from a mollusk and should prove useful for a variety of purposes, particularly genetic mapping and isolation of genes or quantitative trait loci (QTL) affecting disease resistance in the eastern oyster.

Twenty-one single-pair matings of *C. virginica* were made. Tissues from all parents were archived. Juveniles from 14 families were raised at theRutgers Cape Shore Lab. These families have been used to construct second-generation crosses, to provide a two-generation pedigreed oyster archive for genetic mapping studies.

We developed additional nuclear DNA markers to supplement the currently available set of genetic markers.

IMPACTS and/or BENEFITS: Essential tools for genetic improvement of eastern oysters, i.e., DNA libraries, additional genetic markers, and pedigreed families were developed. These will contribute to the long-term goal of developing disease-resistant oysters for population restoration and aquaculture.

PROJECT PUBLICATIONS:

Gaffney, P. M., S. K. J. Allen, and J. Pierce. 1997. Development of nuclear DNA markers and pedigreed families for disease resistance and genetic mapping in the eastern oyster: Progress report. Journal of Shellfish Research 16:257.

Gaffney, P. M., E. A. Orbacz, and Z. Yu. 1998. Using the DCode system to identify DNA sequence variation for studies of population structure in marine organisms. Pp. 4. Bio-Rad.

Wakefield, J. R., and P. M. Gaffney. 1996. DGGE reveals additional population structure in American oyster (*Crassostrea virginica*) populations. Journal of Shellfish Research 15:513.

FUNDING PERIOD: 1995/1996

PROJECT TITLE: Evaluation of American oyster stocks for resource rehabilitation: disease resistance and genetics

PRINCIPAL INVESTIGATOR: Patrick M. Gaffney
AFFILIATION: University of Delaware
CO-INVESTIGATORS and AFFILIATIONS: K. Paynter, D. Meritt, University of Maryland

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PROJECT RESULTS:
Oysters from Texas, Louisiana, Florida, South Carolina, North Carolina, Maryland, Oregon and Delaware were used to make nine hatchery lines, which were deployed at three Chesapeake Bay sites. At two low-salinity sites, negligible disease-related mortality was observed. In Mobjack Bay, significant differences among lines were observed in disease prevalence and mortality.

We surveyed genetic variation in source populations from Canada to Mexico by RFLP and direct sequence analysis of PCR-amplified mitochondrial genes (16S and COI), and developed protocols for analyzing nuclear DNA polymorphisms. Mitochondrial DNA analysis confirmed previous work showing that Gulf Coast and Atlantic oysters are genetically distinct; in addition, we found North and South Atlantic oysters are genetically different, although there appears to be a region of intergradation in the mid-Atlantic.

IMPACTS and/or BENEFITS: This common-garden grow-out experiment demonstrated significant genetic variability for disease resistance and mortality in oysters of different geographic origins. This information can be used for hatchery broodstock selection. In addition, we demonstrated a useful approach for incorporating diverse germplasm into breeding programs, i.e., by crossing males from diverse sources with a common egg pool. This method will be necessary in many cases where viable eggs and sperm cannot be obtained simultaneously from particular populations. Genetic markers developed can be used for broodstock identification, pedigree monitoring, and evaluation of oyster stock enhancement programs.

PROJECT PUBLICATIONS:

(Paynter et al. 1995; Paynter et al. 1997)

Paynter, K.T., P.M. Gaffney, and D. Merritt. 1995. Evaluating eastern oyster stocks for resource rehabilitation. Journal of Shellfish Research 15:517.

Paynter, K.T., P.M. Gaffney, and D. Merritt. 1995. Evaluation of American oyster stocks: Disease resistance and genetics. Journal of Shellfish Research 16:329.

FUNDING PERIOD: 1997-1998

PROJECT TITLE: Physiological and Genetic Correlates of Dermo Disease Resistance in the Eastern Oyster: An Interdisciplinary Approach

PRINCIPAL INVESTIGATOR: Patrick M. Gaffney
AFFILIATION: University of Delaware
CO-INVESTIGATORS and AFFILIATIONS: S. Kaattari, M. Faisal, Virginia Institute of Marine Science

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PROJECT RESULTS:

Ten families of oysters containing high within- and among-family genetic variability were assessed for protease inhibitory (PI) activity against proteases of *Perkinsus marinus*, the pathogen causing Dermo disease. Interrelationships among family survival under field challenge, disease intensity and PI activity were examined. Families with the highest field survival had the lowest body burden of parasite cells, and the highest average PI activity. These results demonstrate the association of increased PI activity with increased disease resistance and higher field survival, and demonstrate that selectable genetic variation for these traits exists in hatchery stocks of eastern oysters.

IMPACTS and/or BENEFITS: These results confirm the feasibility of a selective breeding program for enhancing Dermo resistance, and underscore the importance of broodstock selection in such a program. In addition, they demonstrate the utility of PI activity as a measurable correlate of disease resistance, which can be used in future studies to map the responsible gene(s), and use them or closely linked markers in programs of marker-assisted selection or introgression.

PROJECT PUBLICATIONS:

Faisal, M., S. Kaattari, and P. M. Gaffney. 1998. Physiological and genetic correlates of Dermo disease resistance in the eastern oyster. Journal of Shellfish Research 17:1301.

Oliver, J. L., P. M. Gaffney, J. S K Allen, M. Faisal, and S. L. Kaattari. 2000. Protease inhibitory activity in selectively bred families of eastern oysters. Journal of Aquatic Animal Health 12:136-145.

FUNDING PERIOD: 2000

PROJECT TITLE: Development of a moderate density linkage map of the eastern oyster *Crassostrea virginica*: Identification of disease resistance genes

PRINCIPAL INVESTIGATOR: Patrick M. Gaffney
AFFILIATION: University of Delaware
CO-INVESTIGATORS and AFFILIATIONS: K. Reece, Virginia Institute of Marine Science

PROJECT RESULTS:

Microsatellite marker were designed from enriched genomic DNA libraries and a large random genomic DNA library. Primer sets have been designed to amplify 101 of 336 microsatellite loci identified; 32 amplified well, 19 were screened to detect polymorphisms; 8 have been tested for allelic transmission and Mendelian segregation in four families. Non-Mendelian genotypic proportions were often observed, attributable to null (non-amplifying) alleles and viability selection.

Non-microsatellite nuclear DNA marker development focused on expressed sequence tags (ESTs) and anonymous loci. We tested 32 primer sets and optimized 17 for screening of polymorphisms. Size polymorphisms were found at three intron loci, and restriction enzyme polymorphisms discovered in five loci.

IMPACTS and/or BENEFITS:
The DNA markers developed are being employed for several studies beyond construction of the genetic linkage map, including genetic tracking of oyster restoration projects with selected oyster stocks. In addition, these markers will be applied to high-resolution population genetic studies of natural oyster populations, in order to evaluate the natural distribution of germ plasm diversity.

PROJECT PUBLICATIONS:

Gaffney, P. M. 2001. Genomic approaches to marker development and mapping in the eastern oyster, *Crassostrea virginica*. pp. 84-91 in: (Shimizu, N. et al., ed.) Aquatic Genomics. Springer-Verlag, Tokyo.

Xu, Z., X. Guo, P. M. Gaffney, and J. C. Pierce. 2001. Chromosomal location of the major ribosomal RNA genes in *Crassostrea virginica* and *Crassostrea gigas*. Veliger 44:79-83.

Brown, B. L., D. E. Franklin, P. M. Gaffney, M. Hong, D. DenDanto, and I. Kornfield. 2000. Characterization of microsatellite loci in the eastern oyster, *Crassostrea virginica*. Molecular Ecology 9:2217-2219

W.L. Ribeiro, P.M. Gaffney, S.K. Allen, Jr., and K.S. Reece. Non-Mendelian segregation ratios and null alleles in microsatellite markers of the eastern oyster *Crassostrea virginica*. J. Shellfish Research (submitted)

FUNDING PERIOD: 2001 - 2003

PROJECT TITLE: Cooperative Regional Oyster Selective Breeding (CROSBreed) Project: Comprehensive strategy for genetic rehabilitation and conservation of oysters.

PRINCIPAL INVESTIGATOR: Standish K. Allen, Jr.
AFFILIATION: VIMS, College of William and Mary
CO-INVESTIGATORS and AFFILIATIONS: Eugene Burreson, Mark Camara, Kimberly Reece, Mark Luckenbach, VIMS, College of William and Mary; Patrick M. Gaffney, College of Marine Studies, U. Delaware; Ximing Guo, Gregory A DeBrosse, HSRL, Rutgers; Matthew Hare and Kennedy Paynter, Dept. of Biology, Donald Meritt, Horn Point Environmental Lab, U. Maryland

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PROJECT RESULTS:

Breeding

the Aquaculture Genetics and Breeding Technology Center (ABC) of VIMS has been developing and implementing strategies to genetically invigorate the CROSBreed and DEBY stocks and to produce new ones that build upon previous gains -- namely, line expansion. Line expansion consists of outcrossing the DB and XB lines to other oyster stocks that have demonstrated superior disease resistance when compared to wild control stocks in field experiments: Louisiana Grande Terre (LGT), Tangier Sound and Mobjack Bay (MB). In 2000, the DB line was crossed with MB to produce the DMO line, with LGT oysters to produce the DBLA line, and with the XB line to produce the DXB line. In 2002, under ODRP funding, these 3 lines were perpetuated. In addition, the LGT line was continued. These stocks have been deployed for testing. We expect some "slippage" of the previously realized selective gains, but the augmented genetic variation should promote a rapid recovery and increased scope for long-term improvement in subsequent generations of selection.

Deployment

Much of the restoration effort for Chesapeake Bay oysters is based on reef habitat construction and seeding with natural and selectively bred disease-resistant oyster strains. The extent to which reproduction on seeded reefs will supplement surrounding oyster populations depends on the average age of first reproduction and the geographic extent of oyster larval dispersal. Also, the benefits of genetic enhancement through introgression of disease-resistant alleles into natural stocks depends on the level of inbreeding in selected strains and on the rate of interbreeding between selected strains and natural oysters. We are using the unique genetic signature of disease-resistant strains in Maryand and Virginia subestuaries to measure dispersal from restored reefs the rate of interbreeding and introgression. With cooperation from the Oyster Recovery Partnership and the Chesapeake Bay Foundation we established study populations of DEBY-strain oysters one year ahead of schedule. Genetic analysis of wild adults and juveniles settling near the restored reefs during 2002 has been used to demonstrate better than 95% accuracy in assigning individual oyster recruits to a source population, wild or DEBY-strain. Most benefits from this research are expected in later years as reproductive output of DEBY oysters increases. Ongoing analysis of 2002 spat will indicate to what extent the 2002 DEBY planting in the Great Wicomico River (averaging 6 cm total length) contributed to the relatively high level of recruitment observed.

IMPACTS and/or BENEFITS: ongoing

FUNDING PERIOD: 10/1/99-9/30/01

PROJECT TITLE: Breeding and Evaluation of Oyster (*Crassostrea virginica*) Strains Selected for Resistance to MSX, Dermo and JOD

PRINCIPAL INVESTIGATOR: Ximing Guo
AFFILIATION: Rutgers University
CO-INVESTIGATORS and AFFILIATIONS: Susan Ford, Rutgers University; Gregory DeBrosse, Rutgers University; Roxanna Smolowitz, Marine Biological Lab

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PROJECT RESULTS:
The Northeastern region has a major oyster aquaculture industry that is seriously threatened by three diseases: MSX, Dermo and JOD. We proposed to produce and evaluate the Rutgers NEH strain (selected for MSX- and Dermo-resistance), Flower's FMF strain (selected for JOD), their hybrids, along with a global control (ME, susceptible, from Maine) and local controls that would normally be used by the participating growers. The goal of this study was to breed and evaluate oyster strains for disease resistances and superior growth, and to identify the best performing strain for the NE oyster industry.

The first four groups, NE1-4, were produced at the Rutgers's Cape Shore Facility in June 2000. For each group, two bags of two thousand seed each were deployed at four sites in July 2000: Cotuit Harbor in MA, Milford in CT, Cape Shore and Cape May in NJ. Oysters are cultured in on-bottom or suspended cages. In addition to monthly checks, experimental oysters were sampled five times: 09/00, 12/00, 03/01, 05/01 and 08/01. At each sampling, thirty oysters from each replicate (60/group) were taken for disease analysis and size measurements. No significant levels of JOD were detected. Infection by MSX and Dermo was evident at all four sites. The oysters were about 15 months old as of September 2001, and there was no major mortality due to infection by MSX and Dermo. The disease-caused mortality is expected to start in late 2001 and peak in fall of 2002. Analysis of size data revealed considerable heterosis in growth. The hybrid group (HYB) was larger than both parental stocks by 9 - 19% in shell height and 21 – 40% in total body weight. This project is funded for a third year, and evaluation will continue.

IMPACTS and/or BENEFITS:
Rutgers strains have shown strong resistance to MSX and recently some resistance to Dermo, but not been exposed to JOD. The FMF strain has shown superior growth and markedly improved survival in the face of JOD, but not much resistance to MSX and perhaps neither to Dermo. The hybrids may combine disease-resistance and superior growth from the two selected strains. This study covers the first two years of a three-year project, and most of the disease-inflicted mortality has not occurred. The oysters are only about 15 months old, and it is still too early to make any conclusions. Results so far suggest that the hybrid strain may exhibit heterosis in growth. Fast growth reduces not only culture duration and cost, but also exposure to mortalities. The project is on-going, and oysters will be evaluated to 27 months of age.

PROJECT PUBLICATIONS: (on-going)
Guo, X., S. Ford, G. DeBrosse and R. Smolowitz, 2000. Breeding for a superior eastern oyster for the Northeastern region. J. Shellfish Res., 19(1):572. (abstract)

Guo, X. and J. Kraeuter, 2000. Aquaculture and breeding technology. The Jersey Shoreline, 19(3):1-4.

FUNDING PERIOD: 10/1/01-9/30/03

PROJECT TITLE: Breeding, Evaluation and Molecular Analysis of Oyster Strains Selected for Resistance to MSX, Dermo and JOD

PRINCIPAL INVESTIGATOR: Ximing Guo
AFFILIATION: Rutgers University
CO-INVESTIGATORS and AFFILIATIONS: Susan Ford, Rutgers University; Gregory DeBrosse, Rutgers University; Roxanna Smolowitz, Marine Biological Lab

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PROJECT RESULTS:
This study is a continuation of a previous ODRP project (9915) aimed at breeding and evaluation of oyster strains selected for disease-resistance and superior growth. Part of the study is to continue field evaluation of five strains for a third year. The other part is to use these strains and other families for molecular analysis with the goal of mapping disease-resistance genes.

During the first two years, we produced and deployed the Rutgers NEH strain (selected for MSX- and Dermo-resistance), Flower's FMF strain (selected for JOD), their hybrids, along with a global control (ME, susceptible, from Maine) and local strains that would normally be used by the participating growers. For each strain, two bags of two thousand seed each were deployed at four sites in July 2000: Cotuit Harbor in MA, Milford in CT, Cape Shore and Cape May in NJ. In addition to monthly checks, experimental oysters were sampled 10 times over the past three years: 09/00, 12/00, 03/01, 05/01, 08/01, 11/01, 03/02, 05/02, 08/02 and 11/02. At each sampling, thirty oysters from each replicate (60/group) were taken for disease analysis and size measurements. Dermo infection was heavy at most sites, while MSX and JOD infections were low or absent. At Cape Shore (NJ) where infection was the heaviest, NEH and HYB had the lowest cumulative mortality, 43.5% and 43.6% respectively, compared with 82.3% for FMF, 99.4% for ME and 81.1% for the local control (Delaware Bay wild). In growth, heterosis was no longer statistically significant in most measurement and at most sites. Overall, HYB grew the same as FMF and faster than NEH, while ME and the local controls grew the slowest. The hybrid offered the highest yield by surviving as well as the NEH strain and growing as fast as the FMF strain.

For molecular analysis, we have developed 396 AFLP markers and two linkage maps during the third year. The male map consisted of 114 markers in 12 linkage groups, covering 647 cM. We are also searching for host-defense genes in response to Dermo and MSX infections.

IMPACTS and/or BENEFITS:
This study confirms disease-resistance in the Rutgers strain and the superior growth in the FMF strain. More importantly, results show that the hybrid strain grows as fast as the FMF strain and survives as well as the Rutgers strain. The hybrids are clearly the best choice for aquaculture production. This finding is significant, and the use of the hybrids may bring considerable benefits to oyster farmers in the NE region.

The genetic map produced in this study is the first in the eastern oyster. The moderately dense genetic maps provide a foundation for further mapping other markers and disease-resistant genes. The project is on-going.

PROJECT PUBLICATIONS:

Guo, X., S. Ford, G. DeBrosse and R. Smolowitz, 2003. Breeding and evaluation of eastern oyster strains selected for MSX, Dermo and JOD resistance. Submitted to the 95th Annual Meeting of the National Shellfisheries Association, 2003, New Orleans.

Yu, Z. and X. Guo. 2003. A genetic linkage map for the eastern oyster, *Crassostrea virginica* Gmelin. Biol. Bull., submitted.

Guo, X., Y. Wang, Z. Yu and L. Li, 2002. Physical and linkage mapping in *Crassostrea* oysters. Presented at the World Aquaculture 2002 conference, April 23-28, Beijing, China.
Tanguy, A., S. Ford and X. Guo. 2002. Characterisation of gene expression in response to *Perkinsus marinus* and *Haplosporidium nelsoni* infections in the eastern and pacific oysters. J. Shellfish Res., 21(1):421. (abstract)

Yu, Z. and X. Guo. 2002. A basic AFLP linkage map for the eastern oyster, *Crassostrea virginica* Gmelin. J. Shellfish Res., 21(1):382. (abstract)

FUNDING PERIOD: 9/1/97-8/31/99

PROJECT TITLE: Combined use of a lytic peptide and protease inhibitor: a novel approach to eliminate *Perkinsus marinus* in eastern oysters

PRINCIPAL INVESTIGATOR: Jerome F. La Peyre
AFFILIATION: Department of Veterinary Science, Louisiana State University

CO-INVESTIGATORS and AFFILIATIONS: Richard K. Cooper, Department of Veterinary Science, Louisiana State University; Terrence R. Tiersch, School of Wildlife and Fisheries, Louisiana State Univ.

PROJECT RESULTS:
We identified five protease inhibitors out of ten tested (i.e., AEBSF, chymostatin, "$_1$-antitrypsin, "$_2$-macroglobulin, potato chymotrypsin inhibitor I and II) that decreased *P. marinus* protease activity and inhibited the propagation of the parasite *in vitro*. We found three synthetic lytic peptides out of ten tested (i.e., Phor-21, Agni-21 and Thor-21) that were lethal to *P. marinus*. The protease inhibitors protected the lytic activity of the peptides against degradation by the parasite protease. In addition, none of the lytic peptides and protease inhibitors (except for AEBSF at the highest concentrations) were found to be toxic to oyster cells in primary cultures.

Two protease inhibitors (chymostatin and potato chymotrypsin inhibitor I) were selected to test their effects against *P. marinus* in combination with the lytic peptide phor-21 because of their high inhibitory activity against the parasite, commercial availability, relatively low price, low toxicity to oyster cells, stability and solubility in saline, low molecular size and, availability of synthetic analogues and of gene sequence information. The selected lytic peptide and protease inhibitors were not found to be toxic to hemocytes or to interfere with the hemocytes' host defense related activities (i.e., hemocyte motility, phagocytosis and killing index). Our major finding was that chymostatin alone and Phor-21 with either protease inhibitor significantly reduced the number of parasites in infected hemocytes. These results suggest that entry of the protease inhibitors into infected hemocytes is critical for their activity against *P. marinus* since chymostatin, a low molecular weight protease inhibitor that can readily enter cells, was most effective.

The lytic peptide phor-21, the protease inhibitor chymostatin and a combination of the two were used to treat oysters and their effects on *P. marinus* numbers in hemolymph and whole oysters were determined. Although there was an apparent decrease in *P. marinus* in the hemolymph of oysters injected with chymostatin and chymostatin combined with phor-21 and an apparent increase of parasite in control oysters (saline injected) no significant difference in parasite numbers in hemolymph or whole oysters were found between treatments. The limited dose of chemicals injected and their dilution in the oyster circulatory system as well as the lack of statistical power due to the large standard deviation in parasite numbers may be responsible for the lack of a significant difference.

As part of this study, we also improved two critical techniques to determine the number of *P. marinus* parasites in whole oysters as well as in oyster hemolymph and developed a protocol to isolate and establish primary cultures of eastern oyster hemocytes.

IMPACTS AND/OR BENEFITS
The identification of factors lethal to the oyster parasite *P. marinus* in combination with factors that can inhibit the parasite protease, a putative virulence factor, is a novel approach which can lead to the development of resistant oysters by either genetic manipulation or genetic selection. We used commercially available protease inhibitors and lytic peptides synthesized at LSU to test our original hypotheses and are now in the process of identifying protease inhibitors and lytic peptides found naturally in oyster hemocytes that limit infection by *P.*

marinus. The identification of these defenses against *P. marinus* will provide endogenous genes for developing resistant oysters by increasing gene expression through genetic engineering. Alternatively, the identified factors can be useful as selection markers for breeding resistance to *P. marinus*. This work opens the door to significant future benefits through the genetic improvement of eastern oysters.

PROJECT PUBLICATIONS

La Peyre, J. F., H. S. Kristensen, K. C. McDonough, and R. K.Cooper. 1998. Effects of protease inhibitors on the oyster pathogen *Perkinsus marinus* and oyster cells *in vitro*. #18-2. Third International Symposium on Aquatic Animal Health, August 30 - Sept. 3, 1998. Baltimore, Maryland. Book of Abstracts p. 151.

La Peyre, J. F., K. C. McDonough, and R. K. Cooper. 1998. Killing of the oyster pathogen *Perkinsus marinus* with synthetic antimicrobial peptides *in vitro* and modulation by the pathogen proteases. #S29-2. Third International Symposium on Aquatic Animal Health, August 30 - Sept. 3, 1998. Baltimore, Maryland. Book of Abstracts p. 187.

Kristensen, H. S., J. F. La Peyre, and R. K. Cooper. 1998. Effects of protease inhibitors on the oyster pathogen *Perkinsus marinus in vitro*. American Fisheries Society Meeting, Feb. 4-5, 1998. Bay St. Louis, Mississippi. Book of Abstracts p. 16.

La Peyre, J.F., Smith, A.K. and Cooper, R.K. 1999. Growth inhibition of isolates of the oyster pathogen *Perkinsus marinus in vitro* with protease inhibitors. *In Vitro* Cellular and Developmental Biology 33(3-II):35A.

Coates, G.M., Cooper, R.K. and La Peyre, J.F. 1999. Improvement of the whole-oyster procedure for enumerating *Perkinsus marinus* in oyster tissues. Journal of Shellfish Research 18:328.

Nickens, A. D., and J.F. La Peyre. 2000. Optimization of quantification of *Perkinsus marinus* infections in oyster hemolymph. Aquaculture America 2000, U.S. Chapter, World Aquaculture Society. Feb. 2-5, 2000, New Orleans.

Nickens, A.D., T.R. Tiersch, and J.F. La Peyre. 2000. Effects of a lytic peptide and protease inhibitors on hemocyte functions of eastern oysters. Aquaculture America 2000, U.S. Chapter, World Aquaculture Society. Feb. 2-5, 2000, New Orleans.

La Peyre, J.F. and Li Y. 2000. Isolation and primary culture of eastern oyster hemocytes. Journal of Shellfish Research 19:646.

Nickens, A.D., Tiersch, T.R. and La Peyre, J.F. 2000. Effect of lytic peptide and protease inhibitors on *Perkinsus marinus* in infected hemocytes of eastern oysters. Journal of Shellfish Research 19:647.

Nickens, A.D., Wagner E. and La Peyre, J.F. 2000. Improved procedure to count *Perkinsus marinus* in eastern oyster hemolymph. Journal of Shellfish Research 19:665.

Nickens, A. D., La Peyre, J. F. and Tiersch, T. R. 2001. Treatment of *Perkinsus marinus* infected oyster hemocytes with protease inhibitor chymostatin *in vitro*. Aquaculture 2001, Orlando, FL, January 21-25. Book of Abstracts p. 476.

Nickens, A.D. 2001. Combined effects of a lytic peptide and protease inhibitors on *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*. M.S. thesis, Louisiana State University, Baton Rouge, LA.

Nickens, A. D., La Peyre, J. F., Wagner E. and Tiersch T.R. 2002. Improved procedure to count *Perkinsus marinus* in eastern oyster hemolymph. Journal of Shellfish Research 21:275-732.

FUNDING PERIOD: 10/1/99-9/30/01

TITLE: Optimization of gene delivery for improved oyster health

PRINCIPAL INVESTIGATOR: Richard K. Cooper
AFFILIATION: Department of Veterinary Science, Louisiana State University
CO-INVESTIGATORS and AFFILIATIONS: Terrence R. Tiersch, School of Wildlife and Fisheries, Louisiana State University; Jerome F. La Peyre, Department of Veterinary Science, La. State University

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PROJECT RESULTS:
Significant progress has been made in laying the ground work for the production of transgenic oysters. Many of the tools needed to insure the success of this project have either been completed, or are near completion. Gene delivery techniques have been developed using electroporation (625 v/cm, 25:F, and time constant of 0.3 msec were the optimal parameter tested) and Superfect® (1:3 ratio was optimal) to demonstrate gene transfer into oyster larvae. These techniques demonstrated gene delivery and expression, although expression in larvae was less than 1%. Low expression could be due a number of factors, but most likely is due to failure of the CMV promoter to work efficiently enough to express rsGFP above threshold levels. Detection of rsGFP expression was further complicated by a high degreee of auto-fluorescence in oyster cells that would mask low levels of expression. In an unrelated project, we have demonstrated that accumulation of GFP in cells will inhibit normal growth and lead to higher mortality rates when compared to control cells (Cooper, unpublished data) Due to the difficulties experienced in using rsGFP in oysters, we redesigned our vectors using luciferase under a combination of inducible and constitutive promoters to be used in the continuation of this work funded by GOIP.

To evaluate the potential for gene transfer and expression in adult oysters, two genes were tested: red-shifted green fluorescent protein (rsGFP) and cecropin B (cepB). When oysters were bled at 4 and 10 days post-transfection and PCR was conducted on DNA from oyster hemocytes, the number of animals positive for the genes being tested ranged from 80% to 100%. The results from this project demonstrate the feasibility of DNA delivery to adult animals and the preliminary data supports results obtained from transfected koi and channel catfish (Cooper and Enright, 1999).

IMPACTS AND/OR BENEFITS
The recent problems of the oyster industry associated with *V. vulnificus* have caused a reduced consumer confidence and a decreased demand for oysters. The result has been a decline in prices, which has affected all levels of the Gulf oyster industry. In addition, diseases affecting oysters have the potential for causing declines in available stocks. We are addressing these problems using biotechnology techniques that are becoming routine in other agricultural commodities. Optimum methods for producing and evaluating transgenic oysters are being developed. Specific questions can be answered about gene regulation in oysters and may lead to an all oyster construct or a lytic peptide controlled by an oyster promoter. This project, combined with research in sterilization of oysters may provide the oyster industry with a quality product to overcome negative public opinion and disease problems facing the industry.

PROJECT PUBLICATIONS:

Buchanan, J.T., T.R. Tiersch, and R.K. Cooper. 1999. Gene transfer in oysters. Louisiana Agriculture 42:13.

Buchanan, J.T., Cheng, T.C., La Peyre, J.F., Cooper, R.K. and Tiersch, T.R. 1999. *In vivo* transfection of adult oysters. Journal of Shellfish Research 18:324.

Buchanan, J.T., T. Cheng, J.F. La Peyre, R.K. Cooper, and T.R. Tiersch. 1999. Gene Therapy for Oysters. Louisiana Chapter Meeting of the American Fisheries Society, Baton Rouge, Louisiana, February 4-5. Book of

Abstracts (Best Student Presentation).

Cheng, T., Buchanan, J.T., La Peyre, J.F., Tiersch, T.R. and Cooper, R.K. 1999. Optimization of reverse transcription polymerase chain reaction (RT-PCR) for use with the eastern oyster *Crassostrea virginica.* Journal of Shellfish Research 18:293.

Nickens, A.D., J.T. Buchanan, R.K. Cooper, T.R. Tiersch. 1999. Preliminary Examination of Gene Delivery in Larvae of the Eastern Oyster *Crassostrea virginica.* United States Chapter of the World Aquaculture Society Annual Meeting, Tampa Bay, Florida, January 27-30. Book of abstracts

Buchanan, J.T., A.D. Nickens, R.K. Cooper, and T.R. Tiersch. 2000. Gene Transfer to Eastern Oyster Embryos. United States Chapter of the World Aquaculture Society Annual Meeting, New Orleans, Louisiana, February 2-5. Book of Abstracts

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Buchanan, J.T., T. Cheng, J.F. La Peyre, R.K. Cooper, and T.R. Tiersch. 2000. Transfection of Adult Eastern Oysters by Injection. United States Chapter of the World Aquaculture Society Annual Meeting, New Orleans, Louisiana, February 2-5. Book of Abstracts (Best Abstract Award).

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FUNDING PERIOD:10/1/2001-9/31/2003

TITLE: Accelerated Selective Breeding to Develop Dermo Resistance Oysters in the Gulf of Mexico and Identification of Potential Mechanisms for Increased Disease Resistance

PRINCIPAL INVESTIGATOR: Jerome F. La Peyre
AFFILIATION: Department of Veterinary Science, Louisiana State University
CO-INVESTIGATOR: John E. Supan, Office of Sea Grant Development, Louisiana State University

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PROJECT RESULTS

In summer 2002, two oyster stocks (Grande Terre, Oyster Bayou) which showed high survival rate and low Dermo infection were selected. These oysters (i.e., designated F1 select stocks) are the progeny of large oysters collected from Dermo endemic areas and have undergone three years of field exposure to *Perkinsus marinus*. Control oysters were collected from Snail Bay (Barataria Bay, La) a low salinity area where *P. marinus* prevalence and intensity have historically been low. The F1 select and control oysters were spawned in summer 2002 and their progeny (i.e., designated as F2 select stocks and F1-02 control stock) were grown in up -welling systems at the Louisiana Sea Grant oyster hatchery in Grand Isle, until they reached about 25 mm in length. Oysters (2,500) from each stock were then transferred to floating cages in October 2002 for further grow out. In February 2003, 600 oysters from each F2 select stocks will be inoculated with wild parasites in the laboratory. When oyster mortality reaches 50%, the surviving oysters will be transferred back to floating cages in Grande Isle for field conditioning and will be spawned in summer 2003 to produce F3 select stocks. Control oysters from Snail Bay will be collected and spawned in summer 2003 and their progeny designated as F1-03 control stock. Each year, the mortality, *P. marinus* infection intensity and host defense responses of the remaining oysters of the F2 and F3 select stocks will be compared to control oysters (F1-02 and F1 03) following challenge with the parasite by natural exposure in the field as well as experimental challenge with the parasite in the laboratory.

IMPACTS AND/OR BENEFITS

This project will demonstrate the feasibility of speeding up the process of selective breeding to increase Dermo resistance in Gulf oysters by spawning, growing and challenging oysters to Dermo all within a year. This study is feasible in the Gulf of Mexico because of the high growth rates of oysters raised off-bottom in this subtropical region and because Dermo is the only serious disease affecting oysters in the Gulf, therefore simplifying and potentially enabling rapid selection against this single parasite. Information will also be obtained on the relationship between specific host defense parameters and infection intensities in control and select oyster stocks. Results from this project may validate the use of specific host defense traits as indirect selection criteria to facilitate and further hasten breeding resistance to Dermo in oysters.

PROJECT PUBLICATIONS

FUNDING PERIOD: 1998

PROJECT TITLE: Creation of an oyster cell line in *C. virginica*

PRINCIPAL INVESTIGATOR: Jane C. Burns
AFFILIATION: University of California-San Diego, School of Medicine
CO-INVESTIGATOR and AFFILIATION: Carolyn Friedman, University of California-Davis

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PROJECT RESULTS:
It was the goal of this project to assist the aquaculture industry through creation of a transformed *C. virginica* oyster cell line to facilitate studies focused on isolation and cultivation of oyster pathogens and oyster molecular biology. To achieve this goal, we proposed to develop modified retroviral vectors that contain proto-oncogenes as agents for cellular transformation. The specific goals of the project were:

- to design retroviral vectors that express a variety of proto-oncogenes from appropriate promoter elements that express in oyster cells,
- to infect primary cultures established from both oyster hearts and from enzymatically disrupted early embryos with the transforming retroviral vectors,
- to test resulting cell cultures for immortalization
- to test the cultivation of oyster pathogens in the immortalized oyster cell line

1) Retroviral vectors expressing the SV40 large T antigen and h-ras genes were constructed and used to infect cultured oyster heart cells. Despite PCR evidence that the virus was present in the cells, no expression could be detected by IF staining or Western blotting with appropriate antibodies.

2) Oyster heart infection with retroviral vectors: Infection of cultured heart cells with the pantropic vector expressing luciferase under the control of the LTR promoter yielded detectable luciferase activity that decreased as the cells began dying in culture. Although the dissociated heart cells could be maintained in culture for 1-2 months, the cells appear to stop dividing at the end of the first week in culture.

Hemoylmph analysis: To aid in the formulation of media to nutritionally support oyster cells in culture, we analysed the hemolymph components from *Crassostrea virginica* and *C. gigas*. We measured DNA synthesis in primary cultures incubated in different media and at different temperatures to optimize culture conditions.

Pooled hemolymph (2-3 oysters/pool) was obtained by cardiac puncture. Hemocytes were removed by centrifugation (1,000 x g) and the supernatants stored at -70°C. Analysis of hemolymph components included free amino acids, organic acids, carbohydrates, metals, electrolytes, pH and osmolality. Cultures of heart and embryos were established according to published methods (Boulo et al., 1996 and 2000). DNA synthesis in cultured cells was assessed by ^{3}H-thymidine uptake.Two media formulations based on published data were compared: 1) KS medium (based on Kleinschuster and Swink 1993): L-15 adjusted to 750 mOsm with synthetic sea salts, and supplemented with amino acids, lipids, carbohydrates, vitamins, 10% fetal calf serum (FCS), and 10% *C. virginica* hemolymph, 2) 2X L-15 (Boulo et al., 1996) adjusted to 750 mOsm with NaCl plus 10% FCS.

Selected components that were lower in the media than in the pooled hemolymph samples are shown below:

Table 1. *C. virginica* hemolymph and media analysis expressed as mean values ± S.D; NA= not available.

Component	*C. virginica* hemolymph	KS medium	2X L-15/10%FBS
taurine	195.9±12.1 mg/L	51.9	NA
proline	90.5±3.7	34.2	NA

calcium	40.7±6.2	19.8	9.3
strontium	4.7±0.9	3.0	0.1
boron	4.4±0.7	NA	0.1
zinc	1.3±1.3	NA	0.1

pH ranged from 6.4-6.9.

Different media formulations were made based on the above observations. We used retroviral vector infection of cultured heart cells with a luciferase vector as our assessment of cell division in the culture. It had been noted that luciferase activity following infection with the vector, LLRNL, directly correlated with ^{3}H-thymidine results. Therefore, we examined the possibility of using luciferase activity as a surrogate marker for cell division following vector infection. In the Figure, we have two basal media comparisons (M-6 and M-1) with various additives to M-6. None of the differences were statistically significant, suggesting that none of the different formulations had a dramatic effect on cell division.

Summary: Despite the ability to infect and express foreign genes in cultured oyster cells, we were unsuccessful in creating a *C. virginica* immortalized cell line. The block to cell division in these cultured cells remains unclear. Attempts to manipulate the medium based on analysis of hemoylymph did not result in improved cell division *in vitro*.

Update: Based on these experiences, our group decided to focus on a naturally occurring neoplasia of mussel cells (Mytilus trossulus) as a more promising avenue to a molluscan cell line. This work is currently funded by California SeaGrant (R/A-119). Our first goal in this project is to determine if the hemolymph cells derive from malignant transformation of host mussel cells or whether they are "transplanted" from genetically non-identical methods. We are pursuing molecular markers for the neoplastic cells that will allow us to identify them after transplantation in the laboratory into healthy, recipient mussels.

PROJECT PUBLICATIONS AND PRESENTATIONS:

Boulo V, Cadoret JP, Shike H, Shimizu C, Miyanohara A, Burns JC. Infection of cultured embryo cells of the Pacific oyster, Crassostrea gigas, by pantropic retroviral vectors. In Vitro Cell. Devel. Biol. 36:395-399, 2000.

Burns JC, Shimizu C, Boulo V, Shike H. Pantropic retroviral vectors for gene transfer into invertebrate cells. Presented at the Society for In Vitro Biology, San Diego, CA June 2000; In Vitro Cell. Develop. Biol. 36:12-A, 2000.

Boulo V, Moore JD, Shimizu C, Friedmann CS, Burns JC. Infection of primary cultured cells from two oyster species by pantropic retroviral vectors. Presented at the Society for In Vitro Biology, San Diego, CA June 2000; In Vitro Cell. Develop. Biol. 36:37A, 2000.

Burns JC, Shimizu C, Shike H. Pantropic retroviral vectors for gene transfer in aquaculture species. Presented at the World Aquaculture Society Meeeting, Nice, France, May 2000.

WORKGROUP 3

FRONTIERS IN DISEASE RESEARCH

NOTES

WORKGROUP 3

FRONTIERS IN DISEASE RESEARCH

Characterization

Studies on life cycle stages of the oyster parasite *Haplosporidium nelsoni* (MSX), Susan Ford, Robert D. Barbar and Kathryn A. Ashton-Alcox, April 1, 1991-December 31, 1992.

Life cycle studies of *Haplosporidium nelsoni* (MSX): Spores and non-oyster hosts. Susan Ford, January 1, 1993-March 31, 1995.

Life cycle studies of *Haplosporidium nelsoni* (MSX) using PCR technology. Eugene M. Burreson, Susan E. Ford and Nancy A. Stokes, December 1, 1995-November 30, 1998.

Extracellular proteins from *Perkinsus marinus*: Analysis of pathogenic mechanisms and development of enhanced diagnostics. Mohamed Faisal, Stephen L. Kaatari and Jerome F. LaPeyre, 1995.

The molecular basis for the etiology of the oyster "Dermo" Disease: Gene regulation events susceptible to chemical inhibition. Gerardo Vasta and Adam Marsh, 1995-1996, Dec. 1996-Dec. 1999.

Taxonomic and Genetic Characterization of *Perkinsus marinus*. Development of Mutagenesis and Gene transfer Systems with Application to Therapeutic Strategies. Gerardo Vasta, 1997-1999.

Molecular genetic analysis of *Perkinsus marinus*: Comparisons among laboratory isolates and natural populations. David Bushek, Kimberly Reece and John Graves, Sept. 1997-May 2000.

Intracellular survival of *Perkinsus marinus*: The oxidative stress pathway as a target for therapy. Gerardo Vasta and E.J. Schott, 2001-2003.

The Role of Iron and Host-Derived Growth Factors in Regulating Gene Expression of the Oyster Parasite *Perkinsus marinus*: The Basis for Parasite Proliferation Inhibition Strategies. Gerardo Vasta, 1998-2000.

Detection

Rabbit polyclonal antibody ELISA assay for detection of *Perkinsus marinus* infections in oyster tissues. Christopher Dungan, April 1, 1994-Dec. 31, 1996.

Monoclonal antibody ELISA assay for detection of *Perkiinsus marinus* in oyster tissues. Christopher Dungan, Sept. 1, 1998-Jan 31, 2001.

Relationships between disseminated *Perkinsus marinus* cell abundance, water temperature, salinity, host oyster mortality rate, and dermo disease transmission rate, in Chesapeake Bay waters. Christopher Dungan, Bob Roberson and Eugene Burreson, April 1, 1995-Dec. 31, 1995.

Assessing the Presence and Virulence of "Dermo" Disease in the Environment Using a PCR-Based Diagnostic Assay for *Perkinsus marinus*. Gerardo Vasta and Adam Marsh, 1996-1997.

A molecular approach to Environment studies on *Perkinsus marinus* transmission dynamics of infection in Chesapeake Bay. Gerardo Vasta, 1997-1999.
Epizootiology and pathogenicity of *Perkinsus* species. Gerardo Vasta and J. Robledo, 2001-2003.

Disease-Oyster Dynamics

Identification of inhibitors against *Perkinsus marinus* proteases in oysters. Mohamed Faisal, Steven L. Kaatari and Jerome F. LaPeyre, 1996.

Acid phosphatase(s): a virulence factor of the protozoan parasite, *Perkinsus marinus*, against host oyster's defense? Fu-Lin Chu, Sept 1, 1996-Aug 31, 1997

Comparison of physiological conditions and defense mechanisms among eastern oyster populations with "natural Dermo resistance." Fu-Lin Chu, Oct. 1, 1999-Sept. 30, 2001.

Evaluation of inherent and induced thermal-tolerance in protection of oyster populations from summer mortality caused by Dermo infection and thermal stress. Fu-Lin Chu, Oct. 1, 2001-present.

Induction of potential pathological states in *Perkinsus marinus* by exposure to oyster tissue extracts. Modulation of cell morphology and protease/antigen production. Stephen Kaatari, Kimberly Reece, Eugene Burreson, Oct. 1, 2001-Sept. 30, 2003.

Infectivity, pathogenicity, and epizootiology of the clam parasites *Perkinsus chesapeaki* and *Perkinsus andrewsi* in Chesapeake Bay oysters: Have we been misinterpreting *Perkinsus marinus* epizootiology?

Eugene Burreson, Kimberly Reece, Christopher Dungan and Nancy Stokes, Oct. 1, 2001-Sept. 30, 2003.

Role of oyster lysomsomal enzymes in disease resistance. Fu-Lin Chu and Peter Van Veld, 05/01/1994-12/31/1996

Molecular immune response of the Eastern oyster *Crassostrea virginica* to the parasite *Perkinsus marinus*. Marta Gomez-Chiarri, Oct. 1, 1999-Sept. 31, 2001

Comparative pathogenesis of *P. marinus* disease in bivalves: Development of prevention strategies for *C. virginica.* Robert S. Anderson, September 1, 1997-August 31, 1999.

Antimicrobial peptides: Overlooked mechanisms of disease resistance? Robert S. Anderson, October 1, 2001-September 30, 2003.

Development of nuclear DNA markers and pedigreed families for disease resistance and genetic mapping in the Eastern oyster. Patrick Gaffney, Standish Allen and James Pierce, 1995.

Comparative examination of biochemical correlates of disease resistance in selectively bred *Crassostrea virginica* and *Crassostrea ariakensis*. Gustavo Calvo and Stephen Kaatari, Oct. 1, 2000-Dec. 31, 2001

Interactions of *Crassostrea virginica* hemocytes with the putative etiological agent of juvenile oyster disease (JOD). Katherine Boettcher, Oct. 1, 2001-Sept. 30, 2003

An investigation of the stress response summer mortality and disease resistance *in Crassostrea gigas*. Carolyn Friedman, James Clegg, and Gary Cherr, 1995-1998.
Oyster Herpes Virus Threat to U.S. Oyster Producers. Ralph Elston, Bruce Barber, Eugene Burreson, Carolyn Friedman, Kimberly Reece, Oct. 1, 2001-Sept. 30, 2003

Mortality of the Pacific Oyster, *C. gigas*: Identification and evaluation of multiple environmental stressors and methods to reduce associated mortalities. Daniel Cheney, Ralph Elston, Carolyn Friedman, Gary Cherr, Christopher Langdon, Louis Burnett, Jonathan Davis, Dec. 1, 2001-Sept. 30, 2003

FUNDING PERIOD: April 1, 1991 - December 31, 1992

PROJECT TITLE: Studies On Life Cycle Stages Of The Oyster Parasite *Haplosporidium nelsoni* (MSX)

PRINCIPAL INVESTIGATOR: Susan E. Ford
AFFILIATION: Rutgers University, Haskin Shellfish Research Laboratory
CO-INVESTIGATORS and AFFILIATIONS: Robert D. Barber, and Kathryn A. Ashton-Alcox, Rutgers University, Haskin Shellfish Research Laboratory

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PROJECT RESULTS

Life cycle studies and transmission experiments on *Haplosporidium nelsoni* began soon after the parasite was first identified as the cause of epizootic mortalities of oysters, *Crassostrea virginica*, in the late1950s and early 1960s. One outcome of these studies was that the spore stage, presumed to be a necessary part of the transmission process, was extremely rare in oysters. The general conclusion of these investigations was that an intermediate or alternate host was probably producing the stages that infected oysters, that the oyster might not be a natural host for *H. nelsoni*, and that the supply of infective particles might be totally independent of the supply of oysters.

In this project, we developed information on the significance of sporulation of *H. nelsoni* in young oysters and on the findings of ingested haplosporidan spores in the digestive tract lumina of oysters by intensive sampling of spat and yearling oysters in Delaware Bay during 1991 and 1992 and by examining archived tissue sections for the presence of haplosporidan spores in digestive tract lumina. To introduce new ideas into investigations of the life cycle of *H. nelsoni*, we convened a workshop of experts with knowledge about parasite life cycles, the distribution of other spore or cyst-forming marine organisms, model epizootics, and nearshore ocean circulation, as well as scientists with direct knowledge of the oyster parasite .

Spores of *H. nelsoni* were found from mid May through the third week in July. Over all sampling dates, about 40% of the spat that had advanced plasmodial infections also had spores, although peak prevalence of spores in advanced infections was 75-100%, suggesting that eventually, all advanced infections progress to the spore stage. The highest number of spores in an individual spat was 1.1×10^6, but the overall mean was 1.6×10^5 per individual. The high frequency of sporulation in spat differs greatly from that in older oysters, in which far fewer than 1% of infected individuals have ever been found with spores. Spat are much smaller than adult oysters, with consequently different physiological properties, including a higher metabolic rate; however, whether any of these conditions helps to support sporulation is presently not known.

Ingested haplosporidan spores were found at all locations examined in Delaware Bay, as well as in several other US east coast locations where *H. nelsoni*-infected oysters have been found. They predominated from May through October when they were present in 20% to 40% of the oysters examined. We extrapolated our findings to estimate that there must be several hundred spores per liter in the water filtered by the oysters. Although the spores were present during the infection period for *H. nelsoni*, their frequency showed a weak, negative correlation ($r = -0.55$; $p < 0.02$; $N = 17$ years) with *H. nelsoni* prevalence the following year, suggesting that if they are a stage in the life cycle of that parasite, they are not directly infective to oysters, but may infect an alternate host.

The conclusions of the workshop on the "Life Cycle and Transmission of *H. nelsoni* " are summarized below. Although not all are new, they were refined by the involvement of outside experts and are intended to stimulate future research:

1) It is likely that an alternate or intermediate host, or both, exists for *H. nelsoni*, but we should not give up on looking at the possibility of direct transmission via spores produced in oyster spat (< 1-yr old).

2) It is not surprising that spores would be produced in only one life stage (spat) of the host (oyster); similar occurrences are known in mosquitoes.

3) Because spores are so infrequent in adult oysters, the oyster may be an abnormal/ adventitious host. In this case, an alternate (normal) host would exist that would most likely be very similar to the oyster (i.e., a sessile bivalve) and the seasonal infection cycle would probably also be similar.

4) If an intermediate host exists, it is likely to be quite different from the oyster and possibly one that is itself highly mobile or is dispersed by water currents (i.e., zooplankton, including larval forms). The parasite must have some mechanism to maintain itself near potential hosts (oyster or other similar estuarine species) in the estuary. The potential host is not likely to be a commercially valuable fish species because these have been examined extensively for parasites. Small non-commercial species are candidates, but haplosporidans have never been found in a vertebrate host.

5) An *H. nelsoni* spore produced in another host might not exactly resemble that produced in oysters, but would be in the classified in same phylum. (*Myxobolus cerebralis*, the agent of whirling disease in salmon, for instance, forms two distinct spores in two different hosts: a myxosporean spore in the salmon and a triactinomyxon spore in tubificid worms.) Spore size should not be considered an immutable criterion for differentiating between species.

6) Potential intermediate hosts should match the geographical distribution of *H. nelsoni* and should be producing spores just before oysters become infected. Such intermediate hosts may experience a rapid die-off at this time; also, sporulation may be extensive in the tissues, causing discoloration that may be evident macroscopically or in fresh squashes.

7) Spores could be looked for in water and sediment samples, employing centrifugation and sieving to concentrate appropriate-size particles. Using oysters themselves as trapping and concentrating mechanisms (i.e., search of gut contents) may be the best way.

8) The viability of *H. nelsoni* spores in the environment may not be great because ultrastructurally the attachment of the spore lid does not appear to be very strong. On the other hand, dye exclusion studies suggest that they could be long-lived. Also, spores that settled to the bottom would likely be attacked by bacteria and bottom feeders. Conversely, ingested spores might survive the digestive process or even be made infective in the gut of a vector species, which might also transport them.

9) Previous transmission studies with *H. nelsoni* and *H. costale* spores may have been compromised by lack of freshness of spores. Experience in other in other host-parasite systems indicates that fresh spores are needed for transmission.

10) A detailed, ultrastructural comparison of *H. nelsoni* stages in spat and adult oysters during the sporulation period may provide clues as to why spores are so rarely formed in adults. Comparisons should also be made of physiological differences (e.g., enzyme activities or concentrations of metabolic substrates/products).

11) The use of haplosporidans that regularly produce spores (*H. costale* or *M. teredinis*) as models would be a valuable avenue of research.

12) A combination of settling and filtration of water before passing it over oysters would help determine infective particle characteristics.

FUNDING PERIOD: January 1, 1993 - March 31, 1995

PROJECT TITLE: Life Cycle Studies Of *Haplosporidium Nelsoni* (MSX): Spores And Non-Oyster Hosts

PRINCIPAL INVESTIGATOR: Susan E. Ford
AFFILIATION: Rutgers University, Haskin Shellfish Research Laboratory
CO-INVESTIGATOR and AFFILIATION:

PROJECT RESULTS:
Since its identification as the cause of devastating mortalities of eastern oysters, *Crassostrea virginica*, in the mid-Atlantic beginning in the late 1950s, the causative agent, *Haplosporidium nelsoni*, has been under intensive investigation. Early efforst at defining the life cycle were inconclusive, but suggested the possibility of another host. A group of outside experts attending an ODR-funded workshop on the "Life Cycle and Transmission of *H. nelsoni* " in 1992 urged that a renewed effort be made to search for potential non-oyster hosts and made some suggested as to how to go about this.

The primary objective of this project was to initiate a methodical, but restricted, search for potential alternate or intermediate hosts of *H. nelsoni* by collecting and screening at selected periods and locations a) zooplankters (intermediate host dissimilar to oysters, which could act as a dispersal mechanism for the parasite) and b) a number small bivalve species (alternate host similar to oyster spat in which *H. nelsoni* spores are produced). We also continued our sampling of oyster spat for H. nelsoni spores and our attempts to identify the ingested haplosporidan spores.

Over 1200 individual bivalves and other benthic organisms, and thousands of zooplankters, were collect and examined histologically during the study. Although the species representation was far from equal (e.g., 586 *Tellina* sp. vs 2 *Lyonsia* sp.), it did represent the relative frequency as well as the total abundance of these species at the collection site. Trematodes and cestodes were common in the bivalves and were also found in *Diopatra* sp. and *Balanus* sp. No recognizable protozoans were found in the bivalves and only one copepod and one polychaete were found to have microsporidian infections.

No *H. nelsoni* spores were found in any of the 1500 oyster spat or yearlings examined and, for the most part, total infection prevalence was very low, as it was in other oysters examined during the study period (0 - 30%). We continued to find ingested haplosporidan spores in both fresh and fixed material, but in much lower abundance than in earlier years.

Despite histological examination of several thousand individual potential non-oyster hosts, we did not find any parasites that we could link to *H. nelsoni*. The extraordinarily low prevalence of *H. nelsoni* in oysters in Delaware Bay during the study, even in susceptible groups, indicated that infection pressure (i.e., abundance of infective stages) was minimal. Consequently, interpretation of our negative results is very difficult. A number of scenarios are possible:

1) A non-oyster host was among the species examined, but the low *H. nelsoni* prevalence during the study (as measured in oysters) precluded finding the parasite in this host.

2) A non-oyster host was among the groups (i.e., small bivalves or zooplankters) examined, but was scarce or absent during the study.

3) A non-oyster host exists, but is not among the organisms that we examined.

4) The life cycle is direct, with oyster spat providing the infective stages (spores).

Because our study was not able to verify or eliminate any of the above possibilities, continued efforts must be made to evaluate potential intermediate or alternate hosts for *H. nelsoni*. Zooplankton and small bivalves remain strong candidates. Additionally, the potential role of oyster spat in the parasite's life cycle must be kept in mind.

PROJECT PUBLICATIONS:

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Ford, S. E., R. D. Barber, and E. Marks. 1997. Disseminated neoplasia in juvenile eastern oysters, *Crassostrea virginica*, and its relationship to the reproductive cycle. Dis. Aquat. Org. 28:73-77.

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Powell, E. N., S. E. Ford, J. M. Klinck, and E. E. Hofmann and S. Jordan. 1999. Modeling the MSX parasite in the eastern oyster (*Crassostrea virginica*). III. Delaware and Chesapeake Bay comparisons and the question of transmission. J. Shellfish Res. 18 (2):517-537.

Ford, S.E., Xu, Z. and DeBrosse, G. 2001. Use of particle filtration and UV irradiation to prevent infection by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) in hatchery-reared larval and juvenile oysters. Aquaculture 194:37-49.

Hofmann, E., S. Ford, E. Powell, J. Klinck. (2001). Modeling studies of the effect of climate variability on MSX disease in eastern oyster (*Crassostrea virginica*) populations. Hydrobiologia 460:195-212

Ford, S. E., M.M. Chintala and D. Bushek. 2002. Comparison of *in vitro* cultured and natural *Perkinsus marinus* I. Pathogen virulence. Dis. Aquat. Org. 51:187-201.

Chintala, M.M., D. Bushek and S. E. Ford. Comparison of *in vitro* cultured and natural *Perkinsus marinus* I.I Dosing methods and host response. Dis. Aquat. Org. 51:203-216.

Bushek, D., S. E. Ford and M.M. Chintala. 2002. Comparison of *in vitro* cultured and natural *Perkinsus marinus* III. Fecal elimination and its role in transmission. Dis. Aquat. Org. 51:217-225.

FUNDING PERIOD: 12/1/95-11/30/98

PROJECT TITLE: Life Cycle Studies of *Haplosporidium nelsoni* (MSX) Using PCR Technology.

PRINCIPAL INVESTIGATOR: Eugene M. Burreson
AFFILIATION: Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062

CO-INVESTIGATORS and AFFILIATIONS: Susan E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349 and Nancy A. Stokes, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062

PROJECT RESULTS:

The oyster pathogen *Haplosporidium nelsoni*, also known as MSX, has been the causative agent for oyster mortalities in many areas of the east coast of the U.S. for the past 45 years. Although the distribution and intensity of *H. nelsoni* seem to respond to salinity changes, with increased intensity during drought conditions and decreased intensity during very wet years, the overall virulence and intensity of the pathogen have not decreased since the original epizotics in 1957 and 1959. The presence of this disease is a major obstacle to aquaculture and oyster restoration efforts.

The complete life cycle of *H. nelsoni*, and of all other haplosporidians, remains a mystery. The life cycle stage infective to oysters and the source of that stage have not been identified. Previous attempts to infect oysters directly with *H. nelsoni* spores have been unsuccessful, thus leading to speculation that parasite transmission between oysters occurs via an obligate intermediate host.
The overall objective of this project was to elucidate the complete life cycle of *H. nelsoni* using newly-developed PCR primers specific for this organism. Field samples were collected from both Chesapeake and Delaware Bay and processed for DNA extraction and PCR amplification. PCR-positive samples were subjected to in situ hybridization using a *H. nelsoni*-specific DNA probe and/or DNA sequencing to confirm identity of the amplicon.

Field samples consisted of size-fractionated water (250:m, 75:m, 35:m and 10:m) samples and sediment (aerobic top layer and 250:m) and macroinvertebrates from both locations. Sampling was conducted weekly in 1996, every other week in 1997 and 1998. Total genomic DNA was extracted from each sample and subjected to PCR using *H. nelsoni*-specific primers. Initial difficulties with removal of *Taq* DNA polymerase inhibitors in the environmental samples, especially in sediment, were overcome with ethidium bromide/high salt purification of the DNA and addition of bovine serum albumin to the PCR reactions.

There were very few Delaware Bay samples that yielded *H. nelsoni* PCR product during the course of the project. None of the sediment samples and only four water samples, all in spring/summer 1997, were PCR-positive. Eight samples of macroinvertebrates tested positive for *H. nelsoni*. These included a variety of organisms, such as nereid and spionid worms, dog whelk, gammarid amphipod, and pea crab.

Samples that yielded *H. nelsoni* PCR product were more frequent from the lower Chesapeake than from the Delaware Bay, corresponding to the higher MSX disease prevalence in oysters from this location. PCR-positive results were obtained in many of the Chesapeake water, sediment, and macroinvertebrate samples. Most of the PCR-positive macroinvertebrates were polychaetes, including spionid, nereid, capitelid, and orbiniid worms; however, a variety of other organisms such as gammarid amphipod, isopod, and gastropods also yielded *H. nelsoni* PCR product. Each size fraction of water or sediment was PCR-positive at some point during the study. The only obvious pattern observed was the absence of positive PCR in all samples between late August 1997 and late May 1998 and between late October 1996 and early July 1998 in sediment samples.

Sequencing of the amplicons yielded the *H. nelsoni* sequence in every case, demonstrating that positive samples were not the result of cross-reactivity. In situ hybridizations with the *H. nelsoni*-specific DNA probe were conducted on PCR-positive macroinvertebrate samples to discriminate between true infections and those where *H. nelsoni* simply adhered to the external surface or passed through the gut. Unfortunately, none of the samples used for in situ hybridization revealed *H. nelsoni* cells. We suspect that *H. nelsoni* spores were widely distributed, especially in the Chesapeake sampling location, and that many of the PCR amplicons were from spores either free in the water or sediment or within the guts of organisms. If there were samples with MSX disease, they were probably masked by the abundance of PCR-positive samples from *H. nelsoni* not representing true infections.

IMPACTS and/or BENEFITS:
This project allowed us to optimize and validate the *H. nelsoni*-specific PCR assay for environmental samples. This optimized assay has been used in other laboratories to confirm the presence of *H. nelsoni*. Application of molecular diagnostics to environmental samples was still new at the time of this project and we learned much about the abilities and limitations of PCR assays for large-scale surveys such as this one, and used that information to improve our approach for subsequent life cycle studies.

PROJECT PUBLICATIONS:

Stokes, N. A., B. S. Flores, E. M. Burreson, K. A. Alcox, X. Guo, and S. E. Ford. 1997. Life cycle studies of *Haplosporidium nelsoni* (MSX) using PCR technology. Journal of Shellfish Research 16:336.

Stokes, N. A., B. S. Flores, K. A. Ashton-Alcox, J. R. Pharo, S. E. Ford, and E. M. Burreson. DNA-based molecular diagnostics for *Haplosporidium nelsoni* (MSX) life cycle studies. Abstract for Third International Symposium on Aquatic Animal Health, Baltimore, MD, 30 August–3 September, 1998.

FUNDING PERIOD: 1995

PROJECT TITLE: Extracellular Proteins from *Perkinsus marinus*: Analysis of Pathogenic Mechanisms and Development of Enhanced Diagnostics.

PRINCIPAL INVESTIGATOR: Dr. Mohamed Faisal

AFFILIATION: Department of Environmental Sciences, School of Marine Science/Virginia Institute of Marine Science, The College of William and Mary

CO-INVESTIGATORS and AFFILIATIONS: Drs. S.L. Kaattari and J.F. La Peyre , Department of Environmental Sciences, School of Marine Science/Virginia Institute of Marine Science, The College of William and Mary

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PROJECT RESULTS:

- Axenic cultures were initiated, for the first time, from *Perkinsus marinus*.
- In order to define extracellular products of *P. marinus*, a protein-free chemically defined culture medium was developed.
- It has been demonstrated that *P. marinus* secretes potent chymotrypsin-like serine proteases *in vitro*. These proteases have been identified, isolated, and characterized.
- Extracellular products of *P. marinus* play major roles in the disease initiation and propagation. These products favor the propagation of the protozoan in vivo by suppressing and compromising oyster defense mechanisms.
- Chemotherapeutants with protease inhibitory activities, such as bacitracin, inhibit *P. marinus* propagation *in vitro* and *in vivo.*
- In Dr. Kaattari's lab, a number of monoclonal antibodies were developed. These antibodies recognized *P. marinus* extracellular products *in vivo.*

IMPACTS and/or BENEFITS:

This project has laid the foundation for our current understanding of the major disease processes involved in *P. marinus* infection. Continuous cultures of *P. marinus* became available for the first time and allowed investigations that would have been otherwise impossible to pursue. A number of growth media have been developing thus allowing the biochemical characterization of *P. marinus* extracellular secretions.
The identification of *P. marinus* extracellular products particularly those exhibiting serine protease properties led to the elucidation of pathogenic mechanisms adopted by *P. marinus*. Chemotherapeutants based on protease inhibition appears to be promising and deserves further investigations. A sensitive ELISA assay employing the monoclonal antibodies, developed by Dr. Kaattari, could be successfully applied in epidemiological surveys.

PROJECT PUBLICATIONS:

Ottinger, C.A., T. D. Lewis, D. A. Shapiro, M. Faisal, and S. L. Kaattari (2001): Detection of *Perkinsus marinus* extracellular proteins the tissues of the eastern oyster (*Crassostrea virginica*):Potential use in diagnostic assays. *Journal of Aquatic Animal Health* 13:133-141.

Faisal, M., La Peyre, J.F, E.E. Elsayed, and C.L. Wright (1999). Bacitracin inhibits the oyster pathogen *Perkinsus marinus in vitro* and *in vivo. J. Aquat. Animal Hlth.*11: 130-138.

Faisal, M., D.Y. Schafhauser, K. A. Garreis, E.E. Elsayed and J.F. La Peyre (1999): Purification of *Perkinsus marinus* proteases using bacitracin-sepharose affinity chromatography. *Comp. Biochem. Physiol.* B. 123:417-426.

Tall, B.D., J.F. La Peyre, J.W. Bier, M.D. Miliotis, D.E. Hahes, M.H. Kothary, D.B. Shah, and M. Faisal (1999): *Perkinsus marinus* extracellular protease modulates survival of *Vibrio vulnificus* in eastern oyster (*Crassostrea virginica*) hemocytes. *Appl. Environ. Microbiol.* 65: 4261-4263.

La Peyre, J.F. and M. Faisal (1997): Development of a protein-free chemically defined culture medium for the propagation of the oyster pathogen *Perkinsus marinus*. *Parasite* 4: 67-73.

Garreis, K.A., J.F. La Peyre, and M. Faisal (1996): The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defense parameters *in vitro*. *Fish Shellfish Immunol.* 6: 581-597.

La Peyre, J.F., H. Yarnall, and M. Faisal (1996): Contribution of *Perkinsus marinus* extracellular products in the infection of eastern oysters (*Crassostrea virginica*) *J. Invertebr. Pathol.* 68:312-313.

La Peyre, J.F. and M. Faisal (1996): Optimal culture conditions for the propagation of the oyster pathogen *Perkinsus marinus* (Apicomplexa) in protein deficient medium. *Parasite* 3: 147-153.

La Peyre, J.F. and M. Faisal (1995): *Perkinsus marinus* produces extracellular proteolytic factor(s) *in vitro*. *Bull. Eur. Assoc. Fish Pathol.* 15:28-31.

La Peyre, J.F., D.Y. Schafhauser, E.M. Rizkalla and M. Faisal (1995): Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J. Euk. Microbiol.* 42: 544-551.

La Peyre, J.F. and M. Faisal (1995): Improved method for the initiation of continuous cultures of the oyster pathogen *Perkinsus marinus* (Apicomplexa). *Trans. Am. Fish. Soc.* 124:144-146.

La Peyre, J.F., M. Faisal and E.M. Burreson (1993): *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *J. Euk. Microbiol.* 40:304-310.

FUNDING PERIOD: 1996
PROJECT TITLE: Identification of inhibitors against *Perkinsus marinus* proteases in oysters.

PRINCIPAL INVESTIGATOR: Dr. Mohamed Faisal
AFFILIATION: Department of Environmental Sciences, School of Marine Science, Virginia Institute of Marine Science,The College of William and Mary
CO-INVESTIGATORS and AFFILIATIONS: Drs. S.L. Kaattari and J.F. La Peyre, Department of Environmental Sciences, School of Marine Science/Virginia Institute of Marine Science, The College of William and Mary

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PROJECT RESULTS:

- The plasma of eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters were compared for levels of inhibitory activities against a variety of serine, cysteine, metallo and aspartic proteases representing all protease mechanistic classes including extracellular proteases produced by two oyster-associated pathogens; *Perkinsus marinus* and *Vibrio vulnificus*.
- In comparison to *C. virginica*, *C. gigas* plasma exhibited significantly higher specific inhibition levels (ng protease inhibited/ mu g plasma protein) for papain ($P < 0.001$), pepsin ($P < 0.001$), *P. marinus* protease ($P < 0.001$), trypsin ($P = 0.015$), and *V. vulnificus* protease ($P < 0.001$). Plasma of *C. gigas* did not inhibit the metalloprotease thermolysin.
- Using substrate impregnated gel electrophoresis, it was possible to visualise oyster protease inhibitors against *P. marinus* protease (PMP). *C. gigas* possesses a number of protease inhibitors against PMP with significantly higher specific activities than those in *C. virginica*.

IMPACTS and/or BENEFITS:
Findings of this study demonstrated the presence of protease inhibitors in the plasma of *Crassostrea* spp., which may have an impact upon host defense mechanisms, in addition to other physiological roles. It was obvious that *C. gigas,* which is tolerant to *P. marinus* infection, is capable of mobilizing a larger number of protease inhibitors against PMP with much higher specific activities. This study also gives evidence for the presence of a broad spectrum of humoral host defenses (metalloproteases and their inhibitors) that is brought to bear on *P. marinus* infections by these two *Crassostrea* species.

This newly acquired knowledge is a cornerstone for further investigations on oysters and disease resistance.

PROJECT PUBLICATIONS:

Faisal, M., J.L. Oliver, and S.L. Kaattari (1999): Potential Role of Protease-Antiprotease Interactions in *Perkinsus marinus* infection in *Crassostrea* spp. *Bull. Eur. Assoc. Fish Pathol.* 19:269-276.

Faisal, M., E.A. MacIntyre, K.G. Adham, B.D. Tall, M.H. Kothary, and J.F. La Peyre (1998): Evidence for the presence of protease inhibitors in eastern (*Crassostrea virgininca*) and Pacific (*Crassostrea gigas)* oysters. *Comparative Biochemistry and Physiology* B 121:161-168.

Oliver, J.L., P.M. Gaffney, S.K. Allen, M. Faisal, S.L. Kaattari (2001): Protease inhibitory activity in selectively bred families of eastern oysters, *Crassostrea virginica. Journal of Aquatic Animal Health* 12:136-145.

Oliver, J.L., T. D. Lewis, M. Faisal, and S. L. Kaattari (2000): Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern oyster (*Crassostrea virginica*) and the Pacific oyster (*Crassostrea gigas*). *J. Invertebr. Pathol.* 74:173-183.

FUNDING PERIOD: 1995-6

PROJECT TITLE: The Molecular Basis for the Etiology of the Oyster "Dermo" Disease: Gene Regulation Events Susceptible to Chemical Inhibition

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD
CO-INVESTIGATORS and AFFILIATIONS: Adam Marsh

PROJECT RESULTS:
We obtained support for the hypothesis that SOD is a virulence factor in *P. marinus.*

Elucidation of the effects of host serum and exogenous iron on the expression of superoxide dismutases, actin, protein phosphatase 1:

Host serum induces SOD1 expression.
Elevated iron induces SOD1 expression.
SOD1 expression and actin expression are regulated by growth phase.

Important data were collected on the role of nucleotide metabolism in the proliferation of the parasite in vitro.

Exploration into the possibility that *Perkinsus*, like other intracellular protistan parasites, lacks some of the de novo purine biosynthetic machinery,

IMPACTS and/or BENEFITS:
As a virulence factor, SOD activity would be an appropriate target for the development of antiparasite chemotherapies.

The identification of iron and other as factors that contribute to proliferation opens up the possibility of inhibiting parasite growth. The possibility that exposure to high levels of iron pre-disposes the parasite to virulence.

Disruption of the nucleotide scavenging pathway of human parasites (trypanosomatids, *Toxoplasma*) is a promising strategy for antiparasite chemotherapy.

A thorough understanding of the factors and nutrients that *P. marinus* obtains and requires from the host (oyster) *will both* permit the design of additional intervention strategies, and is essential for understanding its pathogenicity.
Characterization of these first *P. marinus* protein-coding (in addition to rRNA) genes is fundamental for molecular genetic research towards developing interventions, and for elucidating the taxonomic position of the parasite.

PROJECT PUBLICATIONS:
Wright, A.C., Gauthier, J.D., Robledo, J.A.F., Vasta, G.R. Characterization of the actin genes from the oyster parasite *Perkinsus marinus*. In preparation.

Gauthier, J. D. 1998. Development of an in vitro culture system for *Perkinsus marinus*. *Ph. D. dissertation.* University of Maryland at College Park.

Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A. D. M. E., Overstreet, R. M., Porter, J. W., Smith, G. W. & Vasta, G. R. 1999. Emerging marine diseases--climate links and anthropogenic factors. *Science* 285, 1505-10.

FUNDING PERIOD: 1997-99

PROJECT TITLE: Taxonomic and Genetic Characterization of *Perkinsus marinus*. Development of Mutagenesis and Gene transfer Systems with Application to Therapeutic Strategies.

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD

PROJECT RESULTS:
This project saw significant advances in the development of both heterologous (yeast and bacteria) expression of *P. marinus* SOD genes, as well as progress towards the goal of directly transforming *P. marinus* with expression constructs.
Development of tools in this initiative benefited from advances made in previous and concurrently SeaGrant - funded projects (R-ODR/7).

- Improved reporter genes (luciferase, G418 resistance, and enhanced GFP) were tested, and the latter (GFP) used to make fusions with an endogenous *P. marinus* promoter (PmSOD1, which is expressed in all cells).
- We obtained direct evidence that we have introduced DNA into *P. marinus* trophozoites by electroporation. This was the result of modification of culture conditions prior to electroporation as well as optimization of electroporation conditions.
- The information obtained in this initiative led to the construction of a high quality genomic library, from which we have obtained full genomic sequences of more than a half dozen genes.
- Solid media growth conditions were established that should allow for the selection of *P. marinus* mutants in metabolic and oxidative stress pathways.

IMPACTS and/or BENEFITS:
Progress toward the construction of transgenic *P. marinus* will be of great value in many respects, advancing the objective of developing anti-parasite therapies or management strategies. In addition to the tremendous boost to basic research into cell proliferation, structure, and parasite life cycle, it will accelerate discoveries about infection/invasion mechanisms and virulence factors. It will allow the direct testing of hypotheses about virulence factors and infection mechanisms.
The crucial experiments *P. marinus* transformation will make possible include:

- Creation of mutants in pathways of interest to test their effect on disease.
- Promoter-trapping of genes expressed during infection processes.

The value of the genomic library made possible by this project is being repaid many times over as each new gene is clones and characterized.

PROJECT PUBLICATIONS:

Coss, C.A., Robledo, J.A.F., Vasta, G.R. 2001. Fine structure of clonally propagated *in vitro* life stages of a *Perkinsus* sp. isolated from Baltic clam *Macoma balthica. Journal of Eukaryotic Microbiology.* 48: 38-51.

Robledo, J.A.F., Coss, C.A., Vasta, G.R. 2000. Characterization of the NTS, SSU and ITS from the RNA locus of *Perkinsus atlanticus* from Clams (*Ruditapes decussatus*) and development of a species-specific PCR based diagnostic assay. *Jourbal of Parasitology.* 86: 972-978.

Robledo, J.A.F., Coss, C.A., Marsh, A.G., Wright, A.C., Vasta, G.R. 1999. Genetic nucleotide sequence variability in the non-transcribed spacer of the rRNA locus in the oyster parasite *Perkinsus marinus*. *Journal of Parasitology.* 85: 650-656.

FUNDING PERIOD: Sept. 1997 – May 2000

PROJECT TITLE: Molecular genetic analysis of *Perkinsus marinus*: Comparisons among laboratory isolates and natural populations

PRINCIPAL INVESTIGATOR: David Bushek
AFFILIATION: University of South Carolina
CO-INVESTIGATORS and AFFILIATIONS: Kimberly Reece and John Graves, Virginia Institute of Marince Science

PROJECT RESULTS:
This project represented a continuation of a previous ODRP project (NA47FL-0162) in which molecular genetic markers were developed to begin investigating the population genetics of *P. marinus*. **Objective 1** tested ten primer pairs remaining from the previous study and seven new primer pairs were tested, resulting in a total of 23 PCR primer pairs for *P. marinus*. Thirteen produced a single amplification product and eight were polymorphic, making them useful for discriminating strains. All were made available for use in addressing other ODRP priorities. **Objective 2** significantly expanded the collection of *P. marinus* isolates available to the research community from twelve to more than 80. Most have been deposited with ATCC for general distribution as well as nearly 100 clonal cultures of many of the isolates. **Objective 3** determined the genetic similarity of *P. marinus* isolates using the eight polymophic markers from objective 1. No more than two alleles were observed with any marker. Of a potential 6,561 genotypes, only 13 were identified among 162 *P. marinus* cultures examined. Several clonal cultures indicated that individual oysters were infected with more than one strain of *P. marinus*. Genetic analyses indicated that three predominant genotypes were relatively genetically distant. Several genotypes were closely related to one of these common genotypes and two were only observed in clonal cultures. **Objective 4:** To examine the geographic distribution of *P. marinus* strains, the US Atlantic coast was divided into two regions based on the historical and current range of the parasite, with the Gulf of Mexico representing a third region. The distribution of genotypes or genetic strains varied within and among regions. Almost 88% of the isolates possessed one of three predominant genotypes (#1, 3 and 4), but these were not equally distributed among regions. These data indicate that while*P. marinus* is ubiquitously distributed along the Atlantic and Gulf Coasts of the US, different regions possess unique assemblages of genetic strains. Direct isolation of *P. marinus* DNA from infected oysters was also successfully performed using these molecular tools.

IMPACTS and/or BENEFITS:

- Development of strain-specific molecular markers
- Establishment of numerous geographically and genetically distinct isolates and clonal cultures of P. marinus
- Verification of differential distribution of *P. marinus* strains indicating potential risks of moving oyster stock between regions despite presence of other strains.

PROJECT PUBLICATIONS AND PRESENTATIONS:

Reece, K.S., D. Bushek, K.L. Hudson and J.E. Graves. 2001. Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf coasts of the USA, Marine Biology, 139: 1047-1055.

Bushek, D., R. A. Holley and K. S. Reece. 2000. Use of Micromanipulation and "Feeder" Cultures to Clone the Protozoan Oyster Pathogen *Perkinsus marinus*. *J. Eukaryotic Microbiol.*, 47(2):164-166

Reece, K., J. Graves and D. Bushek. 1997. Molecular markers for population genetic analysis of *Perkinsus marinus*. *Mol. Mar. Biol. Biotech.*, 6(3):197-206.

Reece, K.S., K.L. Hudson and D. Bushek. Analysis of genetic variation in *Perkinsus marinus*: Implications for development of DNA-based molecular diagnostics. Oral presentation at the International Conference of the European Association of Fish Pathologists, September 18-23, 1999, Rhodes, Greece

Reece K., D. Bushek and K. Hudson. Molecular genetic studies of *Perkinsus*: examination of inter- and intra-specific variation. World Aquaculture Society Annual Meeting, May 1-7, 2000. Nice, France.

Reece, K.S. D. Bushek and K. Hudson. 1999. Analysis of the geographic distribution of *Perkinsus marinus* strains. Nat'l Shellfisheries Assoc. Ann. Mtg., Halifax, Nova Scotia. Apr. 18-22, 1999. *J. Shellfish Res.* 18(1):320

Reece, K.S., K.L. Hudson and D. Bushek (1999) Analysis of genetic variation in strains of the oyster pathogen *Perkinsus marinus*. Oral presentation, 24th Annual Eastern Fish Health Workshop. March 9-12, 1999, Atlantic Beach, NC.

Reece, K.S. , J. E. Graves, D. Bushek and K. Hudson. Analysis of genetic variation in the oyster pathogen *Perkinsus marinus*. 2nd International Conference on Shellfish Restoration. Hilton Head, SC. November 1998., *J. Shellfish Res.*, 17(4):1311-1312.

Reece, K. S., D. Bushek, K. Hudson, and J. Graves. Molecular genetic analysis of *Perkinsus marinus*, a protozoan pathogen. 3rd International Symposium on Aquatic Animal Health. Baltimore, MD. August 1998

Bushek, D., Reece, K., Graves, J., Holley, R. and Hudson, K. Development of molecular markers for population genetic analysis of *Perkinsus marinus*. SERRS Spring Meeting. Athens, GA. March 1998

FUNDING PERIOD: 2001-03

PROJECT TITLE: Intracellular Survival of *Perkinsus marinus*: the Oxidative Stress Pathway as a Target for Therapy.

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD
CO-INVESTIGATORS and AFFILIATIONS: E. J. Schott

PROJECT RESULTS:
We are characterizing potential targets for anti-parasite therapies in the antioxidant pathway of *P. marinus*. These are:

- Ascorbate peroxidase, an activity that appears to be unique to the parasite.
- Two distinct iron superoxide dismutases, PmSOD1and PmSOD2, that have unique cellular localizations. PmSOD1 in the mitochondrion, and PmSOD2 in an unknown compartment located below the plasma membrane.
- Structural information, obtained by X-ray crystallographic analysis of both PmSOD1 and PmSOD2, will be used to explore the design of structure-based chemotherapeutic agents.
- We are also actively characterizing the antioxidant repertoire of the oyster host, to evaluate how to specifically inhibit the anti-oxidant pathway of the parasite.

We have found that the parasite is highly sensitive to hypochlorite, but not to superoxide or hydrogen peroxide. Hypochlorite is the product of host MPO activity.

IMPACTS and/or BENEFITS:
These results advance the search for effective anti-*P. marinus* chemotherapies.

- Many successful anti-parasitic chemotherapies consist of oxidative-stress inducing compounds that are more toxic to parasite than host. Chemotherapies can be improved by a thorough understanding of oxidative stress mechanisms in both host and parasite.
- The verification that the mitochondrion is the location of a putative *P. marinus* virulence factor (FeSOD), synergizes with the fact that in related human parasites, therapies that inhibit mitochondrial function are highly effective.
- We have advanced in our goal to inhibit the ROS removal pathway of *P. marinus,* by direct enzymatic inhibitors, and disrupt the function, maturation, or subcellular localization of these enzymes. Our collaborations with experts in molecular modeling will facilitate discovery of these compounds.
- In closed systems, application of chemotherapies would be an excellent choice for production of clean seed.
- Knowledge that hypochlorite kills *P. marinus* suggests that a search for oysters with robust MPO activity may uncover a source of resistance to infection.

PROJECT PUBLICATIONS:
Schott, E.J., Robledo, Pecher. W.M., Okafor, F. and Vasta, G.R. Resistance of the protistan parasite *Perkinsus marinus* to reactive oxygen intermediates. In preparation for *Journal of Experimental Parasitology*

Robledo, J.A.F., Vasta, G.R. Gene characterization of Slc11a transporter in the protistan parasite *Perkinsus marinus*. In preparation for *Molecular and Biochemical Parasitology*.

Coss, C. A. 2000. Investigation of *Perkinsus* species from clams sympatric to oysters, with emphasis on infections in baltic clams *Macoma balthica* of Chesapeake Bay. Ph. D. Dissertation, The George Washington University, Washington, D.C.

FUNDING PERIOD: 1998-00

PROJECT TITLE: The Role of Iron and Host-Derived Growth Factors in Regulating Gene Expression of the Oyster Parasite *Perkinsus marinus*: The Basis for Parasite Proliferation Inhibition Strategies

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD

PROJECT RESULTS:

- Recombinant expression and purification of PmSOD1 and PmSOD2 in *E. coli.* Proof that both enzymes genes encode iron-cofactored SODs (FeSODs).
- Demonstration that expression of PmSOD1 in yeast causes an FeSOD to be targeted to the mitochondria. This novel finding highlights the potential for PmSOD1 to be a target for intervention.
- This 'proof of principle' of *functional* heterologous expression of *P. marinus* genes shows that a library-based approach, to discover new antioxidant genes, is feasible.
- Establishment of conditions that allow the growth of *P. marinus* as microcolonies. This will enable design of mutagenesis screens to identify P. marinus mutants deficient in metabolic or antioxidative pathways.

IMPACTS and/or BENEFITS:

The crystallization and structure determination of recombinant proteins is a prerequisite for developing structure-based inhibition strategies.

Recombinant expression of SODs ultimately led to (1) the crystallization of pure enzymes, and (2) generation of specific antisera against both SODs, and their use in immunofluorescence to discover:

- The novel localization of the FeSOD to a subcellular compartment suggests that the parasite mitochondrion may itself represent a target for intervention.

Creation and testing of specific mutants in antioxidant pathways is crucial to understanding how this potential virulence-related pathway can be used as a target for intervention.

PROJECT PUBLICATIONS:

Gauthier, J. D. & Vasta, G. R. 2002. Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite *Perkinsus marinus. Journal of Experimental Zoology* 292, 221-30.

Wright, A., Ahmed, H., Gauthier, J., Silva, A. & Vasta, G. 2002. cDNA cloning and characterization of two iron superoxide dismutases from the oyster pathogen Perkinsus marinus. *Moleculat and Biochemical Parasitology.* 123,73-7

Gauthier, J. D. & Vasta, G. R. Effects of iron on expression of antioxidant genes of the protistan parasite *Perkinsus marinus*. In preparation.

FUNDING PERIOD: 4/1/94 – 12/31/96

PROJECT TITLE: Rabbit polyclonal antibody ELISA assay for detection of *Perkinsus marinus* infections in oyster tissues

PRINCIPAL INVESTIGATOR: Christopher F. Dungan
AFFILIATION: Maryland Dept. of Natural Resources, Oxford Laboratory, Oxford, MD
CO-INVESTIGATORS and AFFILIATIONS: none

PROJECT RESULTS:

Two polyclonal rabbit antisera to *Perkinsus marinus* cellular immunogens were produced, purified, specificity-tested, and labeled.

Using biotinylated and unconjugated preparations of the same rabbit IgG antibodies, we designed, optimized, and tested the clinical performance of an immuno-affinity, antigen-capture, microplate ELISA assay for quantitative detection of *P. marinus* antigens in heterogeneous oyster tissue homogenates.

The optimized ELISA assay format, which utilized a biotin/streptavidin-complex detection system, quantitatively detected as little as 10ng of *P. marinus* protein in oyster tissue homogenates (40:g of oyster protein), with assay absorbance signals proportional to parasite protein concentrations in spiked oyster tissue homogenates.

The optimized ELISA assay reliably and quantitatively detected *P. marinus* infections in clinical samples categorized to harbor light, moderate, and heavy infections by standard Ray's fluid thioglycollate medium (RFTM) assays, and ELISA assay signals were linearly correlated with categorical infection intensities estimated by paired RFTM solid tissue assays.

The optimized ELISA assay failed to detect *P. marinus* infections in 3% of oysters that were independently diagnosed as infected by RFTM assays of replicate tissue sub-samples (false-negative errors), but yielded positive diagnoses for 81% of oysters diagnosed as uninfected by RFTM assays. The latter differential reflects the increased sensitivity and accuracy of the ELISA assay over the standard RFTM assay.

IMPACTS and/or BENEFITS:

Results validated a newly developed diagnostic assay for rapid, sensitive, quantitative, and automated detection of *P. marinus* infections in oyster tissues.

Diagnostic results from ELISA are available in hours, compared to days for existing assays, and reduced analytical effort per analyzed sample may reduce diagnostic costs, concurrent with increasing the speed and accuracy of dermo disease diagnostic assays.

Assay antibodies have been liberally distributed to diverse oyster disease research investigations, and have also been distributed for commercial diagnostic assay development.

PROJECT PUBLICATIONS:

Dungan C.F. and Hamilton R.M. 1997. Microplate ELISA assay for detection of *Perkinsus marinus* in oyster tissues. *J. Shellfish Res.* 16:330-331 (abstract)

FUNDING PERIOD: 9/1/98 – 1/31/01

PROJECT TITLE: Monoclonal antibody ELISA assay for detection of *Perkinsus marinus* in oyster tissues

PRINCIPAL INVESTIGATOR: Christopher F. Dungan
AFFILIATION: Maryland Dept. of Natural Resources, Oxford Laboratory, Oxford, MD

PROJECT RESULTS:
Stable hybridomas secreting murine IgG_1 monoclonal antibodies (MAB) to *Perkinsus marinus* epitopes were produced, cloned, cryopreserved, and archived under liquid nitrogen refrigeration at two locations

Ten murine IgG_1 MABs were sub-isotyped, produced (5mg) by hybridoma culture, chromatographically purified, and their binding epitopes characterized on western blots of solubilized and electrophoretically-resolved *P. marinus* proteins. Five MABs bound to primary structure epitopes of single 57-59kD reduced proteins. One MAB labeled a primary structure epitope present in both 31kD and 28kD reduced proteins. One MAB (16C11) labeled a ubiquitous, recurrent, primary structure epitope on >13 reduced proteins of 14-66kD sizes. Three MABs labeled secondary structure epitopes on unreduced proteins, which were not labeled on reduced proteins in western blot assays.

Binding specificity testing with MAB immunoassays of histological sections of aquatic hosts infected by diverse parasites, including different *Perkinsus* species, were thwarted by the failure of 9/10 of our MABs to label *P. marinus* in histological sections from infected oysters. Like our prior rabbit polyclonal antibodies to *P. marinus*, MAB 16C11 specifically and intensely labeled formalin-fixed *P. marinus* and *Perkinsus* sp. cells in histological sections from infected oysters, clams, scallops, and abalone, did not label *Haplosporidium nelsoni* cells in oyster sections, but did label *Hematodinium* spp. parasitic dinoflagellates infecting three decapod crustacean species.

Due to our inability to validate mono-specificity for *P. marinus* in any of our MABs, diagnostic ELISA assay development was abandoned pending future production of MABs with more robust binding epitopes that are unique to *P. marinus*, or collection of a suite of infected tissue samples from diverse hosts and parasites, that are suitable to determining MAB binding specificities in non-histological assays.

IMPACTS and/or BENEFITS:
Results confirm the feasibility of producing MABs to *P. marinus* epitopes, using antigens extracted from axenic in vitro parasite isolate cultures as both immunogens and hybridoma screening assay antigens, and confirm the utility and shortcomings of several assay types for screening and characterization of such MABs. These results will inform future efforts.

If convenient histological specificity testing assays are included in future diagnostic MAB production efforts, formaldehyde treatment of immunogens, but not of hybridoma screening assay antigens, may result in selection for cloning of hybridomas secreting MABs that bind to epitopes present in both fixed (histological) and unfixed (host tissue homogenate) samples.

Benefits of MAB-based diagnostic immunoassays include the perennial availability of characterized, specific antibody reagents from immortal hybridoma cell lines. This MAB attribute reduces assay reagent costs, enables assay specification standardization and precision, and promotes the commercial production of rapid, low cost, high-sensitivity immunossay systems.

PROJECT PUBLICATIONS:
Dungan C.F. and Hamilton R.M. 2001. Production and binding specificities of MABs to P*erkinsus marinus* cellular antigens. *J. Shellfish Res.* **20**:543 (abstract)

FUNDING PERIOD: 4/1/95 – 12/31/95

PROJECT TITLE: Relationships between disseminated *Perkinsus marinus* cell abundance, water temperature, salinity, host oyster mortality rate, and dermo disease transmission rate, in Chesapeake Bay waters

PRINCIPAL INVESTIGATOR: Christopher F. Dungan
AFFILIATION: Maryland Dept. of Natural Resources, Oxford Laboratory, Oxford, MD
CO-INVESTIGATORS and AFFILIATIONS: Dr. Bob S. Roberson, University of Maryland, College Park, MD; Dr. Eugene M. Burreson, Virginia Institute of Marine Science, Gloucester Point, VA

PROJECT RESULTS:
Employed new flow cytometric immunoassays of high-frequency water samples collected in dermo disease-endemic Chesapeake Bay waters at Oxford, MD and Gloucester Point, VA to estimate seasonal disseminated *Perkinsus marinus* cell abundances in environmental waters, and inter-annual variability in timing and magnitudes of dermo disease infection pressure peaks.

Found that conservatively estimated peak disseminated parasite cell abundances varied from 3,000 – 12,000 cells/liter between sampling sites, and between years at each sampling site.

Found that peak parasite abundance timing varied annually during July and August at the VIMS site, and between June and August at the Oxford site.

Found that peak host oyster mortality rates both closely preceded and closely followed peak parasite abundances at the VIMS site, but consistently followed peak parasite abundances at the Oxford site, suggesting that significant parasite dissemination regularly precedes peak mortality of infected hosts, and may also follow mortality peaks.

Found that maximum new infection acquisition rates by uninfected sentinel oysters deployed and retrieved biweekly at the VIMS site coincided temporally with periods of high measured parasite abundances, but that infection incidences and parasite abundances were not statistically correlated due to infrequent infection of sentinel oysters during periods of low parasite abundance. Highest dermo disease transmission rates occurred during periods of high disseminated parasite abundances, and low transmission rates persisted during periods of low parasite abundances.

Found that earliest lesions in newly infected laboratory and environmentally exposed oysters, occurred most frequently in external epithelia and connective tissues of the gills and mantle, and less frequently in gut epithelia. The importance former

IMPACTS and/or BENEFITS:
Results validated a new technical method that was developed for estimating real-time dermo disease infection pressure, by analysis of environmental water samples.

The proposed mechanism for dermo disease transmission as following death and decomposition of infected oysters was supported, and was expanded by evidence suggesting that significant parasite dissemination also precedes host mortality.

Oyster resource management strategies for avoidance of dermo disease transmission were informed by direct measurements of the seasonality and relative intensities of infection pressures in high- and moderate-salinity environments, and of interannual variations in both timing and intensity of infection pressures at individual sites.

Known avenues through which *P. marinus* infects oyster hosts were expanded to include non-gut portals that do not require pathogen ingestion, as significant invasion sites.

PROJECT PUBLICATIONS:

Ragone Calvo L.M., Dungan C.F., Roberson B.S., and Burreson, E.M. *submitted* A systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in the lower Chesapeake Bay. *Dis. Aquat. Org.*

Ragone Calvo L.M., Burreson, E.M., Dungan C.F., and Roberson B.S. 1996. *Perkinsus marinus* transmission dynamics in Chesapeake Bay. *J. Shellfish Res.* **15**:496 (abstract)

Dungan C.F., Hamilton R.M., Ragone Calvo L.M., and Burreson E.M. 1996. Identification of *Perkinsus marinus* portals of entry by histochemical immunoassays of challenged oysters. *J. Shellfish Res.* **15**:500 (abstract)

FUNDING PERIOD: 1996-7

PROJECT TITLE: Assessing the Presence and Virulence of "Dermo" Disease in the Environment Using a PCR-Based Diagnostic Assay for *Perkinsus marinus*

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD
CO-INVESTIGATORS and AFFILIATIONS: A. Marsh

PROJECT RESULTS:

- Validation of the first species-specific PCR assay for "Dermo".
- Demonstration of the application of *P. marinus* -specific molecular technology for the assessment of populations of *P. marinus* in oysters along the eastern seaboard.
- Highly sensitive detection of *P. marinus* in oysters, including:
    - Tissue specific detection, particularly in hemolymph sampled from live oysters.
- Sequence-based clarification of the taxonomic status of *P. marinus* with respect to other protists (dinoflagellates, apicomplexans).

IMPACTS and/or BENEFITS:
The long and short term benefits to both management and essential research are manifold, and include:

- The species-specific PCR methodology made possible the identification of additional species of *Perkinsus* in oysters and clams along the eastern seaboard.
- Rapid and sensitive detection of cryptic infections in hatchery stocks, including the potential to detect infections ~ 1 month earlier than by the FTM method.
- Rapid and routine certification of disease free oyster spat from hatcheries
- Detection of infections / verification of disease free status in animals to be used for research.
- Taxonomic relationships are crucial for understanding and predicting parasite behavior, based on related, well-understood protists.

PROJECT PUBLICATIONS:

Marsh, A., Wright, A. C. & Vasta, G. R. 1996. Isolation and characterization of marker genes for *Perkinsus marinus*. *Journal of Shellfish Research* 15: 516. Conference 88. Annu. Meeting of the National Shellfisheries Association, Baltimore, MD (USA), 14-18 Apr 1996

Robledo, J.A.F., Coss, C.A., Gauthier, J.D., Wright, A.C., Vasta, G.R. 1998. Species-specificity and sensitivity of the polymerase chain reaction-based assay for the detection of the parasite *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica.* A comparative study with the fluid thioglycollate medium assay. *Journal of Parasitology.* 84: 1237-1244.

Elandalloussi, L.M., Leite, L.M., Afonso, R., Nunez, P.A., Robledo, J.A.F., Vasta, G.R., Cancela, M.L. Development of a PCR-ELISA assay for diagnosis of Perkinsus marinus and *Perkinsus atlanticus* infections in bivalve mollusks. *Marine Biotechnology.* In review.

Coss, C. A. 2000. Investigation of *Perkinsus* species from clams sympatric to oysters, with emphasis on infections in baltic clams *Macoma balthica* of Chesapeake Bay. Ph. D. Dissertation, The George Washington University, Washington, D. C.

FUNDING PERIOD: 1997-99

PROJECT TITLE: A Molecular Approach to Environment Studies on *Perkinsus marinus*. Transmission Dynamics of Infection in Chesapeake Bay.

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD

PROJECT RESULTS:

- Validation that the PCR detection method is more sensitive than FTM methodology, and detects infections earlier in the season than FTM.
- Confirmation that there are at least two major genotypes of *P. marinus*, (Type I and II), and the finding that they have differential geographic distribution.
- Validation of a quantitative PCR method of Dermo detection.
- Application of the PCR methodology to study *P. marinus* reservoirs in other estuarine species and sediment.

IMPACTS and/or BENEFITS:

- Unlike FTM, detection of *P. marinus* by PCR methodology is highly sensitive for all life stages: trophozoite, hypnospore, zoospore.
- Earlier detection of Dermo infections will allow improved monitoring and management of oyster stocks
- Species-specific detection will allow false positives for *P. marinus*, possible with the FTM method, to be avoided.
- Major savings to seed producers with non-*P. marinus* species of *Perkinsus* that would not be able to market their product if FTM were the sole criterion for assessment of Dermo.
- Quantitative, species-specific detection of *Perkinsus* spp will allow close monitoring of experimental field trials and laboratory experiments.
- Detection of *P. marinus* and other *Perkinsus* spp in possible reservoir hosts and sediment represents a significant

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PROJECT PUBLICATIONS:

Schott, E.J., and Vasta, G.R. The tubulin gene family of *Perkinsus marinus*. In preparation for *Journal of Experimental Parasitology*

Robledo, J.A.F., Schott, E.J., Marsh, A. G., Vasta, G.R. A preliminary survey of Perkinsus marinus genes using expression sequence tags (EST). In preparation for *International Journal for Parasitology.*

Robledo, J.A.F., Pecher, W.T., Delwiche, C., Vasta, G.R. Phylogenetic analysis of *Bonamia ostreae* based on the SSU rRNA and actin sequences, and development of a PCR-based diagnostic assay. In Preparation.

FUNDING PERIOD: 2001-03

PROJECT TITLE: Epizootiology and pathogenicity of *Perkinsus* species.

PRINCIPAL INVESTIGATOR: G. R. Vasta
AFFILIATION: Center of Marine Biotechnology, Baltimore, MD
CO-INVESTIGATORS and AFFILIATIONS: J.A.F. Robledo

PROJECT RESULTS:

- Sampling throughout summer and fall months of 2002.
- Eleven sites, four areas with both *C. virginica* and *M. mercenaria* sampled.
- Over 1800 individuals sampled: *C. virginica, M. mercenaria, M. arenaria, G. demissa.*
- To date, over 1200 PCR analyses conducted: Generic (*Perkinsus*), *P. marinus,* and *P. andrewsi* specific. Strain-specific analysis of *P. marinus* also conducted on selected amplicons.
- Preliminary data shows high prevalence of *P. marinus* in oyster at several sites, with moderate prevalence in others. In *M. mercenaria, P. marinus* prevalence lower.
- Dual infections (oyster) with both *P. marinus* and *P. andrewsi* in more than one site sampled.
- An indication that both type I and type II strains of *P. marinus* may infect a single individual oyster
- At least two individuals testing positive with the generic primer set did not generate products with either *P. marinus* or *P. andrewsi* primers , suggesting that an additional *Perkinsus* sp. was present. Generic amplicons from these individuals are being sequenced.

IMPACTS and/or BENEFITS:
Having developed the highly specific and sensitive molecular tools to discern which species of *Perkinsus* is present in an individual, we are now applying them to field situations.

These results improve significantly on the FTM method of assessing *Perkinsus* sp prevalence. They confirm suspicions that:

- not all *Perkinsus* infections are due to *P. marinus*
- more than one than one species or strain can infect an individual.

Additional information such as that provided in this study will help managers of oyster stocks along the east coast. For example, *Perkinsus* infections shown to be due to a non-*P. marinus* species may pose less of a threat to oysters, and thus be managed differently, from those *P. marinus* infections of known pathogenicity.

There may be additional, as yet undescribed, species of *Perkinsus* along the Atlantic coast.

PROJECT PUBLICATIONS:

Coss, C.A., Robledo, J.A.F., Ruíz, G.M., Vasta, G.R. 2001. Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of species-specific PCR-based diagnostic assay. *Journal of Eukaryotic Microbiology.* 48: 52-61.

Robledo, J.A.F., Nunez, P.A., Cancela, M.L., Vasta, G.R. 2002. Development of an *in vitro* clonal culture and characterization of the rDNA locus of *Perkinsus atlanticus*, a protistan parasite of the clam *Tapes decussatus. Journal of Eukaryotic Microbiology.* 49: 414–422.

Pecher, W.T., Robledo, J.A.F., Vasta, G.R. Identification of an additional rRNA fragment encoded by *Perkinsus andrewsi* genome. In Preparation.

FUNDING PERIOD: 1996

PROJECT TITLE: Identification of inhibitors against *Perkinsus marinus* proteases in oysters.

PRINCIPAL INVESTIGATOR: Dr. Mohamed Faisal
AFFILIATION: Department of Environmental Sciences, School of Marine Science/Virginia Institute of Marine Science, The College of William and Mary
CO-INVESTIGATORS and AFFILIATIONS: Drs. S.L. Kaattari and J.F. La Peyre, Department of Environmental Sciences, School of Marine Science/Virginia Institute of Marine Science, The College of William and Mary

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PROJECT RESULTS:

- The plasma of eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters were compared for levels of inhibitory activities against a variety of serine, cysteine, metallo and aspartic proteases representing all protease mechanistic classes including extracellular proteases produced by two oyster-associated pathogens; *Perkinsus marinus* and *Vibrio vulnificus*.
- In comparison to *C. virginica*, *C. gigas* plasma exhibited significantly higher specific inhibition levels (ng protease inhibited/ mu g plasma protein) for papain ($P < 0.001$), pepsin ($P < 0.001$), *P. marinus* protease ($P < 0.001$), trypsin ($P = 0.015$), and *V. vulnificus* protease ($P < 0.001$). Plasma of *C. gigas* did not inhibit the metalloprotease thermolysin.
- Using substrate impregnated gel electrophoresis, it was possible to visualise oyster protease inhibitors against *P. marinus* protease (PMP). *C. gigas* possesses a number of protease inhibitors against PMP with significantly higher specific activities than those in *C. virginica.*

IMPACTS and/or BENEFITS:
Findings of this study demonstrated the presence of protease inhibitors in the plasma of *Crassostrea* spp., which may have an impact upon host defense mechanisms, in addition to other physiological roles. It was obvious that *C. gigas,* which is tolerant to *P. marinus* infection, is capable of mobilizing a larger number of protease inhibitors against PMP with much higher specific activities. This study also gives evidence for the presence of a broad spectrum of humoral host defenses (metalloproteases and their inhibitors) that is brought to bear on *P. marinus* infections by these two *Crassostrea* species.
This newly acquired knowledge is a cornerstone for further investigations on oysters and disease resistance.

PROJECT PUBLICATIONS:

Faisal, M., J.L. Oliver, and S.L. Kaattari (1999): Potential Role of Protease-Antiprotease Interactions in *Perkinsus marinus* infection in *Crassostrea* spp. *Bull. Eur. Assoc. Fish Pathol.* 19:269-276.

Faisal, M., E.A. MacIntyre, K.G. Adham, B.D. Tall, M.H. Kothary, and J.F. La Peyre (1998): Evidence for the presence of protease inhibitors in eastern (*Crassostrea virgininca*) and Pacific (*Crassostrea gigas)* oysters. *Comparative Biochemistry and Physiology* B 121:161-168.

Oliver, J.L., P.M. Gaffney, S.K. Allen, M. Faisal, S.L. Kaattari (2001): Protease inhibitory activity in selectively bred families of eastern oysters, *Crassostrea virginica*. *Journal of Aquatic Animal Health* 12:136-145.

Oliver, J.L., T. D. Lewis, M. Faisal, and S. L. Kaattari (2000): Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern oyster (*Crassostrea virginica*) and the Pacific oyster (*Crassostrea gigas*). *J. Invertebr. Pathol.* 74:173-183.

FUNDING PERIOD: 09/01/96 to 08/31/97

PROJECT TITLE: Acid phosphatase(s): a virulence factor of the protozoan parasite, *Perkinsus marinus*, against host oyster's defense?

PRINCIPAL INVESTIGATOR: Fu-Lin E. Chu
AFFILIATION: Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062

PROJECT RESULTS:
The objectives of the one-year project were to biochemically and physiologically characterize *Perkinsus marinus* acid phosphatase and other antioxidant enzymes and determine the effects of temperature and salinity on the acid phosphatase secretion from *P. marinus*. During the one-year funding period, we determined 1. The extra- and intra-cellular antioxidant enzymatic activities in different strains of *P. marinus*; 2. Effect of temperature and salinity on the secretion of acid phosphatase from *P. marinus*; and 3. The intra-cellular and distribution of acid phosphatase in *P. marinus*. The extra- and intra-cellular activities of acid phosphatase (AP), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GP) were examined in six different *P. marinus* strains/isolates, i.e., Delaware Bay, New Jersey (DB-NJ), Mobjack Bay, Virginia (MB-VA), Barataria Bay, Louisiana (BB-LA), Laguna Madre, Texas (LM-TX), Oxford, Maryland (OX-MD), and York River, Virginia (YR-VA). It was found that no calatase or GP was detected in *P. marinus* meront cells and the extra-cellular product (ECP). The YR-VA strain had significantly higher extracellular AP activities (units/mg cell protein) than all other strains. Intracellular AP activity was low (<1.0 unit/mg total cell protein) in all strains. LM-TX strain had the greatest intracellular AP activity. The mean SOD activity (ng SOD/mg total cell protein) was higher in the YR-VA strain, but statistically insignificant from the other strains. SOD activity was detected only in the culture media of 97 days old *P. marinus* culture. The effects of temperatures (4, 12, 20, and 28°C) and osmolality (400, 570, 840 mOsm/kg; equivalent to 14, 20, and 28 ppt) were investigated. AP secretion by *P. marinus* meronts was found to be cell density dependent and increased with increased culture temperatures and cell proliferation. AP secretion was similar in *P. marinus* cultured at 400 and 570 mOsm/kg media, but higher than *P. marinus* cultured at 870 mOsm/kg media. Results of the ultrastructural study revealed that intense AP activity was in the nucleus of the parasite. Based on its distribution in the nucleus, AP may be playing a role in events leading to cell cycle regulation. Although, it is still unclear whether AP in *P. marinus* plays a role in suppressing the host defense, it was discovered that no production of reactive oxygen intermediates (ROIs), toxic metabolites, was elicited when oyster hemocytes were challenged with *P. marinus*. Additionally, chemiluminescence (a measurement of ROI production) evoked from zymosan (yeast)-stimulated hemocytes was suppressed (reduced) when treated with live *P. marinus* meronts and ECP.

IMPACTS and/or BENEFITS:
Results of the study provide important information for a better understanding of the biochemistry, physiology and the virulence of the oyster parasite, *Perkinsus marinus*. It was a first study on the characterization of acid phosphatase and potential antioxidant enzymes in *P. marinus* and their roles in interfering and modulation of host defense. Via this study, it was first discovered that *P. marinus* cells and ECP were capable of suppressing host respiration burst, production of reactive oxygen intermediates, although the exact mechanism behind the ROI suppression by *P. marinus* remains to be elucidated. Results of the study have been presented in both international and national professional/scientific conferences/workshops and reported in two publications in renowned scientific journals. Currently an additional manuscript is in preparation and will be submitted for publication.

PROJECT PUBLICATIONS:
Volety, A. K. and F.-L. E. Chu. 1997. Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the American oyster, *Crassostrea virginica*. J. Parasitol., 83:1093-1098.

Volety, A. and F.-L. E. Chu. 1995. Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite, *Perkinsus marinus*. Dev. Com. Immunol. 19:135-142.

FUNDING PERIOD: October 1, 1999 to September 30, 2001.

PROJECT TITLE: Comparison of physiological condition and defense mechanisms among eastern oyster populations with "natural Dermo resistance"

PRINCIPAL INVESTIGATOR: Fu-Lin E. Chu
AFFILIATION: Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

PROJECT RESULTS:

The objectives of the project were to compare the disease resistance in putative "natural Dermo resistant (NDR) and non-resistant" populations deployed in Chesapeake Bay and determine whether Dermo tolerance/resistance is a result of enhanced growth characteristics or specific defense parameters. To achieve these objectives, stocks representative of putative NDR and disease susceptible populations were selected and spawned. The progenies from these stocks were deployed and raised in two Dermo-enzootic sites of the Chesapeake Bay from September 1999 to December 2001. These stocks included: two NDR from Chesapeake Bay (Rappahannock River, CRB; Tangier Sound, CTS), two NDR from Gulf of Mexico, Louisiana (Grande Terre, LGT; Oyster Bayou, LOB), one susceptible control from Chesapeake Bay (Choptank River, CCR) and Louisiana (Hackberry Bay, LHB), and a positive control hatchery resistant strain (CROSBreed, XB, 9 generation for MSX, 4 generation for Dermo). Over the two-year study, oysters were sampled monthly or bimonthly from deployed stocks to determine Dermo infection, mortality, growth, cellular and humoral defense-related activities (plasma protease inhibition activity, plasma lysozyme activity, number of circulation hemocytes, ability of hemocytes to kill *P. marinus*) and physiological condition (condition index, tissue protein, lipid and glycogen content). Variation in growth and survival occurred among these natural stocks. The CRB stock grew fastest, reaching market size (3") within 18 months, but had the highest Dermo prevalence and intensity and lowest survival at both deployed sites (average ~10% at two sites). Generally, Chesapeake stocks had higher levels of Dermo infection than Louisiana stocks. Despite higher Dermo intensity than Louisiana stocks and similar to CRB, CTS consistently had lower mortality in both deployed sites. The CTS stock's disease tolerance (MSX and Dermo) and growth performance is comparable to the hatchery disease resistant strain, XB, with slightly better survivorship (~60%) than XB (50%). The LOB stock had a survivorship of 50% when exposed to Dermo alone and grew to the largest size over all stocks (3.5-4.0"). However, although Louisiana stocks had lower Dermo infection, results from the second year suggest that the LOB stock was less tolerant to cold climate and more susceptible to MSX. These results provide evidence that natural disease resistance does exist. Condition index varied seasonally and was related to variation in glycogen content. These parameters were similar among stocks and did not correlate strongly with degree of infection. The measured cellular and humoral defense-related parameters did not show any correlation among individuals within a oyster stock and between oyster stocks, and with Dermo infection and mortality over time.

IMPACTS and/or BENEFITS:

Via the funding of this project, we identified a natural disease resistant Chesapeake Bay stock (Tangier sound, CTS) which showed comparable disease tolerance and growth performance to the hatchery disease resistant strain (XB). Results of our study further demonstrated that Louisiana stocks are Dermo tolerant. Based on our findings, the VIMS Aquaculture Genetics and Breeding Technology Center (ABC) crossed Louisiana oysters with the XB hatchery strain in 2001, to increase resistance to Dermo and MSX, which will significantly benefit the burgeoning aquaculture industry in the Chesapeake Bay. Presently, Middle Peninsula Aquaculture sells oyster seed produced by Tangier Sound brood stock and Chesapeake Bay Aquaculture is using Louisiana and Tangier Sound brood stock for commercial oyster production. Cooperation with Regent Point and Port Kinsale marinas have resulted in further research projects continuing at these sites. Finally, through a series of meetings, workshops, and collaborative seminars, we have disseminated information to members of non-commercial oyster gardener associations across Virginia, as well as the scientific community. Additionally, to disseminate results, numerous papers (a total of 7) had been presented at international and national conferences. Several manuscripts are currently in preparation and will be submitted for publication.

PROJECT PUBLICATIONS:

Encomio, V.G., S. Stickler, F.-L. E. Chu. 2002. Evaluation of physiological condition in Dermo resistant oysters. J. Shellfish Res. 21: 373.

Stickler, S., V. Encomio, S. K. Allen, and F.-L. E. Chu. "Natural Dermo resistance" in eastern oyster stocks: Chesapeake studies and defense-related activities. J. Shellfish Res. 21:376.

Stickler, S. M., V. G. Encomio, F.-L. E. Chu, and S. K. Allen. 2000. Growth, mortality, and defense against Perkinsus marinus in eastern oysters, *Crassostrea virginica*. J. Shellfish Res. 19:666-667.

Encomio, V. G., S. M. Stickler, and F.-L. E. Chu. Energy reserves in *Perkinsus marinus* infected and uninfected oysters. J. Shellfish Res. 19: 662.

Encomio, V., S. Stickler, F.-L. E. Chu and S. Allen (In preparation). Physiological condition and energy reserves associated with Dermo infection and mortality in natural Dermo tolerant/resistant and susceptible oyster stocks. J. Shellfish Res.

Stickler, S.M. V.G. Encomio, J.F. LaPeyre, S.K. Allen, Jr., F.-L. E. Chu. (In preparation). Defense-related activities associated with natural Dermo resistance. J.Shellfish Res.

FUNDING PERIOD: 10/ 01/01 to present

PROJECT TITLE: Evaluation of inherent and induced thermal-tolerance in protection of oyster populations from summer mortality caused by Dermo infection and thermal stress

PRINCIPAL INVESTIGATOR: Fu-Lin E. Chu
AFFILIATION: Virginia Institute of Marine Science, College of William and Mary

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PROJECT RESULTS
Increased Dermo and MSX proliferation coupled with thermal stress may be dually responsible for high oyster mortalities in the summer and fall months. Heat shock proteins (hsps) are known to protect organisms at the cellular level from thermal or other environmental stress. Heat shock proteins are also believed to play a role in protecting many animals from pathogenic insult and to be involved in immune functions and host-pathogen interactions, in addition to conferring tolerance to thermal and other environmental stresses. Induced hsp expression may increase tolerance to diseases by reducing the stress of high temperatures associated with seasonal parasitic bouts, direct protection from the effects of parasitism, or both.

In this project we are examining the roles of thermotolerance and heat shock proteins in resistance to Dermo in the eastern oyster. Our objectives are to 1. characterize the heat shock protein (hsp) expression in Chesapeake Bay natural disease resistant (Tangier sound, CTS) and susceptible (Rappahannock River, CRB) stocks identified via our previous project (VA-OD-99-3/5-29456), 2. compare the thermal tolerances and hsp expression in disease resistant and susceptible stocks, 3. compare *in situ* seasonal hsp expression in these oyster stocks, and 4. determine whether induced hsp expression confers tolerance to thermal stress and *P. marinus*. In earlier experiments we found that heat shock protein expression remained elevated over controls (oysters maintained at ambient temperature) for up to two weeks following a sublethal heat shock. Exposure of oysters to sublethal heat shock increases tolerance to a subsequent lethal heat treatment (induced thermal tolerance). We are currently testing whether induced thermal tolerance and associated hsp expression confers enhanced tolerance to parasitic stress in oysters challenged with isolated *P. marinus*. We are also examining intraspecific variation in thermal tolerance and hsp expression among genetically distinct oyster stocks. Initially, we compared thermal tolerance (LT50) between two oyster stocks found previously to be Dermo resistant (CTS and Oyster Bayou, LA – LOB). Both stocks were identical in age and reared under identical conditions. Thermal tolerance was higher in CTS oysters than LOB oysters, although hsp levels were similar. Changes in hsp isoform expression are being examined to resolve how isoform variation may affect total hsp levels. Our previous results show that two constitutive isoforms are present in gill tissue of eastern oysters. A third inducible isoform has been observed by other scientists' studies of west coast *C. gigas* and *C. virginica*, so further investigation in hsp expression patterns is warranted. Seasonal variation in hsp expression is also being compared among Dermo resistant (F2 CTS and XB) and susceptible (F2 CRB) oyster stocks deployed in the field. Initial results show little variation in hsp levels despite distinct differences in survival among these stocks. High survivorship occurred in the F2 CTS and XB stocks despite high Dermo infection. Survival in F2 CTS and XB oysters were similar to each other and their respective parental stocks, suggesting that Dermo resistance is heritable. The F2 CRB stocks experienced high mortality, identical to the F1 CRB stock.

IMPACTS and/or BENEFITS:
As shown by the results from this on-going project, resistance to Dermo is heritable as patterns of survival are retained from F1 to F2 generations in CTS stock. Oyster stocks from this and earlier research are presently being utilized in the selective breeding program at VIMS and by commercial aquaculture enterprises (Middle Peninsula Aquaculture and Chesapeake Bay Aquaculture), but little is known about how these oysters respond to natural stress. The interaction of thermal stress and parasitism can significantly affect oysters' survival. Heat shock proteins may be a key component in oysters' tolerance to thermal and parasitic stress. Improving the stress

tolerance of disease resistant oysters may lead to development of improved oyster strains for both aquaculture and restoration purposes. Our research will identify mechanisms involved in stress tolerance in oysters, which will aid strain development. Results from this project have applications to both the hatchery and grow out stages of oyster aquaculture production, which could result in combining strain selection with optimal culture techniques to maximize aquaculture yields. Results from this project will be presented at the 2003 National Shellfisheries Association meeting, and will be prepared for publication.

PROJECT PUBLICATIONS:

Encomio, V.G.; Chu, F.L.E. The Role of Heat Shock Proteins in Tolerance to Parasitic Stress in the Eastern Oyster, *Crassostrea virginica.* National Shellfisheries Association Annual Meeting, April 13-17, 2003, New Orleans, USA (upcoming invited presentation)

PROJECT NUMBER: NA16RG1697 FUNDING PERIOD: 10/01/01-09/30/03

PROJECT TITLE: Induction of potential pathological states in *Perkinsus marinus* by exposure to oyster tissue extracts. Modulation of cell morphology and protease/antigen production.

PRINCIPAL INVESTIGATOR: Dr. Stephen L. Kaattari, Professor
AFFILIATION: Virginia Institute of Marine Science

CO-INVESTIGATORS and AFFILIATIONS: Dr. Kimberly S. Reece, Assistant Professor, Virginia Institute of Marine Science; Dr. Eugene M. Burreson, Professor, Virginia Institute of Marine Science

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PROJECT RESULTS:
(Project is in progress)
Objective I: Determine the nature of the induced *P. marinus* molecules, and develop specific serological and molecular probes for these molecules.

Status: This objective builds upon the results from previous ODRP grant research regarding the alteration in cellular differentiation and expression of potential virulence factors by inclusion of oyster products in the cell culture medium. The isolation of differentially expressed genes by subtractive hybridization using a *P. marinus* cell line grown in the presence or absence of oyster products is proceeding. We are currently in the process of screening the cDNA libraries, and it is expected that several differentially expressed genes will be identified. In conjunction with the molecular subtractive techniques, subtractive serological probes are also being developed. Briefly, mice are immunized with the extracellular products (ECP) of *P. marinus* grown in unsupplemented medium, the B-lymphocytes which are reactive to constituents of that ECP are then deleted using cyclophosphamide, and the mouse is re-immunized with ECP from cells previously grown in supplemented medium. This technique is expected to skew the immune response toward those antigens which are only present in the latter immunogen. Initial attempts at the technique were unsuccessful, due to a generally poor immune response of the mice toward the ECP. Effort was directed at enhancement of the immunogenicity of the ECP preparations, and it has now been determined that ECP not only contains intrinsic factors which are immunosuppressive in the mouse, but that an immunosuppressive media component was being co-concentrated with the ECP proteins during processing. Development of a purification and concentration technique has subsequently allowed production of a panel of IgG isotype monoclonal antibodies directed against components of unsupplemented ECP. Concurrently, the subtraction protocol has been refined using a highly immunogenic protein mixture, *C. virginica* hemolymph. These refinements are now allowing the use of subtractive immunization for detection of upregulated proteins.

Objective II: Examine various *C. virginica* stocks to determine whether there is an intra-species correlation between relative resistance and the ability to induce protease expression. Status: The original proposal listed several groups of oysters which were to be supplied by the VIMS oyster hatchery. Unfortunately, there were high mortalities in several of the groups, and the remaining groups have remained very small under quarantine conditions. This has necessitated acquisition of several groups of oysters which have been previously deployed in the field. Since this experiment involves comparison of body burdens after experimental infection, we have elected to proceed with this experiment during late winter, when the naturally occurring infection levels will be at their lowest. At that time, we will gradually acclimate the oysters to warmer water and proceed with this phase of the experiment.

Objective III: Homogenates from distinct oyster tissues will elicit varying degrees of serine protease expression and parasite differentiation. Status: Initial results have shown that there are distinct patterns of cell differentiation in cultures supplemented with homogenates of different organs. There has not, however, been consistent induction of the low molecular weight proteases that are postulated to be virulence factors. We are

currently in the process of repeating this experiment, using oysters from a different geographic area. We are also supplementing the cultures at a higher protein level. Previous studies have demonstrated a dose-responsive elicitation of the low molecular weight protease when using whole oyster homogenate and oyster hemolymph. The dose range for any single organ is not known, and the initial experimental dose may have been under the critical limit for induction.

Objective IV: Purify, isolate, and identify oyster molecules that induce low molecular weight protease expression in *P. marinus*. Status: Oyster homogenate and hemolymph are extremely complex and apparently have a tendency to react with sugar moieties present on typical commercial size exclusion chromatographic beads. Initial attempts to broadly separate the components of oyster products into size classes was frustrated by aggregation of the proteins, and their resultant passage through the column in the void volume. Several different buffers have been used, and it has been found that inclusion of magnesium can reduce the amount of aggregation. We are currently attempting size separation using an acrylamide-based matrix, which should reduce the interaction of the oyster proteins with the column. Additionally, we have optimized a fractionation technique using preparative isoelectric focusing for use in the event that size exclusion becomes untenable, or that we need further resolution of a particular size fraction.

Objective V: Determine the effect of culture supplementation on *P. marinus* infectivity (reversal of *in vitro* induced attenuation): Status: We currently have 7 groups of *P. marinus* cells which are being grown in media supplemented with both high and low concentrations of tissue homogenates from oyster strains and species of varying susceptibility to *P. marinus* infection. After four weeks of culture, these cells will be used to infect *C. virginica* oysters, and the degree of infection will be determined by enumeration of total parasite body burden four weeks after infection. In addition, we will at that time assess the induction of the low molecular weight proteases by each of these oyster groups and assess correlation between infectivity and ability to upregulate protease expression.

IMPACTS and/or BENEFITS:
Until all of the results can be assessed at the end of the grant period, it is difficult to project the total impact. We have already succeeded in production of antibody probes to *P. marinus* ECP, which has been an elusive goal in *P. marinus* research. In addition, the recognition that *P. marinus* ECP contains intrinsic immunosuppressive factors in the murine model leads to interesting path of investigation as to the role of specific ECP components in the modulation of oyster defenses.

PROJECT PUBLICATIONS:

Earnhart, C. and Kaattari, K. Development of serological techniques to host-induced proteins of the oyster parasite, *Perkinsus marinus*. Poster. Presented to the International Symposium on Aquatic Animal Health, 2002, New Orleans, LA.

Earnhart, C. and Kaattari, K. Submitted. The murine humoral response to *in vitro* generated parasite antigens is critically diminished by the Pluronic F-68 block copolymer, a defined media component.

FUNDING PERIOD: 10/01/01 – 09/30/03

PROJECT TITLE: Infectivity , pathogenicity, and epizootiology of the clam parasites *Perkinsus chesapeaki* and *Perkinsus andrewsi* in Chesapeake Bay oysters: have we been misinterpreting *Perkinsus marinus* epizootiology?

PRINCIPAL INVESTIGATOR: Eugene M. Burreson[1]
CO-PIs: Kimberly S. Reece[1], Christopher F. Dungan[2], Nancy A. Stokes[1]
AFFILIATIONS:[1]Virginia Institute of Marine Science, College of William and Mary, [2]Maryland Department of Natural Resources, Oxford Cooperative Lab

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PROJECT OBJECTIVES AND RESULTS:
The overall objectives are to determine the infectivity and pathology of *P. chesapeaki*and *P. andrewsi* to oysters and to use that information to reevaluate our understanding of *P. marinus* epizootiology in oysters.

Year 1: Develop specific DNA probes and PCR primers for *P. marinus*, *P. chesapeaki* and *P. andrewsi* based on ITS or LSU sequences of the ribosomal RNA gene complex.

Year 2: Determine infectivity and pathogenicity of *P. chesapeaki* and *P. andrewsi* to oysters with laboratory challenge experiments. Determine prevalence and intensity of *P. chesapeaki* and *P. andrewsi,* as compared to *P. marinus*, in selected oyster and clam populations in Chesapeake Bay.

PROJECT RESULTS YEAR 1 and YEAR 2 (current year): The internal transcribed spacer region (ITS) of the ribosomal RNA gene complex was chosen for the design of species-specific PCR primers for Chesapeake Bay *Perkinsus* species. Uncloned and clonal cultures of three *Perkinsus* isolates were sequenced at the ITS region. One isolate was from *Mya arenaria*, the type host for *P. chesapeaki*, and two were from the razor clam, *Tagelus plebeius*. Our ITS sequence data showed two clades within a single monophyletic group, one clade containing the GenBank-deposited *P. chesapeaki* sequence, the other clade containing the GenBanked sequence for *P. andrewsi*. Sequences obtained from individual clonal cultures from both hosts were found in both clades suggesting that *P. chesapeaki* and *P. andrewsi* are the same species. Large subunit rRNA sequences (LSU) were also determined to confirm the findings based on ITS sequences. Clonal cultures isolated from *M. arenaria* and *T. plebeius* were used and once again the *Perkinsus* sequences from both hosts grouped into a monophyletic clade. To further test whether these two species were the same, the previously developed *P. andrewsi*-specific primers (Coss et al. 2001b) were tested with these same clonal isolates and they amplified the DNA from all of the cultures. Due to the evidence suggesting that *P. chesapeaki* and *P. andrewsi* are the same species, one PCR primer set was developed to specifically amplify these ITS sequences, and one in situ hybridization probe was designed to target the LSU region. An in situ hybridization probe specific to *P. marinus* was also designed based on the LSU region sequences. The PCR primers were optimized with cultures and are currently being optimized for use with infected host materials. Real-time/quantitative PCR was optimized with *Perkinsus* genus-specific primers in water samples during year one and will continue to be optimized with infected host material during the second year.

Oyster beds located adjacent to a variety of clam species were sampled during the first year throughout the Chesapeake Bay in order to determine prevalence and intensity of *P. marinus* and *P. chesapeaki/andrewsi* in oyster populations. Specifically, 7 sites were sampled in the Maryland portion of the Chesapeake Bay for *Crassostrea virginica* and *M. arenaria*. *T. plebeius* was also sampled at 5 of the 7 sites in MD. *Macoma balthica* was found at only one site in MD with 7 individuals. In the Virginia portion of the Chesapeake Bay, there were 3 sampling sites; *C. virginica* and *M. mercenaria* were collected from 2 sites and *C. virginica*, *M. balthica* and *T. plebeius* were collected from 1 site. All samples were assessed for *Perkinsus* prevalence by Ray's fluid thioglycollate medium (RFTM) assay, processed for histology, and the gill was processed for use in DNA analysis. The RFTM assay showed greater than 80 percent prevalence of *Perkinsus* in all *C. virginica* samples except one MD sample with 52 percent prevalence. Prevalence of *Perkinsus* in the clam species by RFTM was usually very high prevalence, with a few exceptions. High prevalence of *Perkinsus* infections was found in all of the *M. arenaria* samples, except for one sample from MD. In addition, *Perkinsus* was found at a high prevalence in all but one of the *T. plebeius* samples. *Perkinsus* was found in *Macoma balthica* in both states, however, only one *M. mercenaria* sample was found to have *Perkinsus* infections and only at a 3 percent prevalence. The DNA samples are currently being extracted for screening by PCR. The histology sections are paraffin embedded and awaiting results of the PCR analysis before the final processing for in situ hybridization analysis.

IMPACTS and/or BENEFITS: The thorough DNA sequencing clonal cultures of *Perkinsus* species isolated in Chesapeake Bay in order to develop PCR primers has elucidated significant intra-specific variation within clonal cultures at the ITS locus. ITS sequences previously thought to be either *P. chesapeaki* or *P. andrewsi*- specific were found simultaneously within the genome of several clonal cultures. This observation suggests that *P. chesapeaki* and *P. andrewsi* should be synonymized. Sequence data from several clonal *Perkinsus* cultures isolated from various hosts, including *M. balthica*, and from several different geographic locations in the Chesapeake Bay, as well as sequences of other genes were collected for this study and further support synonymization. These observations suggest that intra-, as well as inter-specific, sequence variation needs to adequately characterized before designing molecular diagnostics and to use molecular data to support descriptions of new species of *Perkinsus,* and other pathogens. The field studies in this project are beneficial to further understand the epizootiology of *P. marinus* on oysters. It will help to indicate whether the assumption that positive results from RFTM assays as only *P. marinus* infections has been accurate, or whether some infections are actually caused by other *Perkinsus* species. Furthermore, this field study will be useful in comparing the RFTM diagnosis with PCR analysis and quantification.

PROJECT PUBLICATIONS:

Dungan, C.F., R.M. Hamilton, K.L. Hudson, C.B. McCollough and K.S. Reece. (2002) Two Epizootic Infectious Diseases in Chesapeake Bay Commercial Clams *Mya arenaria* and *Tagelus plebius*. Dis. Aquat. Org. 50:67-78.

Reece, K.S. (2002) Utilization of Molecular Genetic Data for Detection, Identification and Description of *Perkinsus* Species. .Oral presentation at the National Shellfisheries Association Conference April 14-18, 2002, Mystic, CT. Abstract in J. Shellfish. Res. 21(1):376.

Dungan, C.F., R.M. Hamilton, C.B. McCollough, K.S. Reece, and K.L. Hudson. (2002) Epizootic diseases in Chesapeake Bay clams. Oral presentation at the National Shellfisheries Association Conference April 14-18, 2002, Mystic, CT. Abstract in J. Shellfish. Res. 21(1):372.

FUNDING PERIOD: May 1, 1994 to December 31, 1996.

PROJECT TITLE: Role of oyster lysosomal enzymes in disease resistance

PRINCIPAL INVESTIGATOR: Fu-Lin E. Chu
CO-INVESTIGATORS: Peter Van Veld.

AFFILIATIONS: Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062

PROJECT RESULTS:
The objectives of the project were to elucidate the role of oyster lsosomal enzymes in defense against the parasite, *Perkinsus marinus* and determine the effects of temperature and salinity on lysosomal enzyme activities in oysters. During the funding period, we performed experiments to: 1. determine the synergetic effects of temperature and salinity (3, 10, 20 ppt at 10 and 25°C) on extracellular and intracellular lysosomal enzyme activities in oysters, 2. compare the extracellular and intracellular lysosomal enzyme activities of oysters from high salinity and low salinity habitats, 3. determine if release of lysosomal enzymes occurs when oyster hemocytes are challenged with *P. marinus* infective particles, 4. isolate and partially purify lysozyme from oyster plasma, 5. determine the effects of partially purified lysozyme on the proliferation and function of *P. marinus*. The following summarizes the results from the above experiments. Both temperature and salinity affected the activities of plasma and hemocytes, but no consistent pattern was observed in the examined enzymes (lipase, acid phosphatase, L-aminopetidase) except lysozyme. While there was no interaction effect of temperature and salinity on plasma lysozyme activity, the interaction of temperature and salinity significantly affected the hemocyte lysozyme activity in oysters. Generally, no significant temperature and salinity effects were noted on lysosomal enzyme activity in oyster tissues. Oysters collected from the low salinity habitat of the Chesapeake Bay demonstrated significantly higher lysozyme activity in plasma and gill-mantle tissues than oysters collected from the high salinity habitat. Apparently the gill and mantle are sensitive to changes in temperature and salinity. When hemocytes were challenged with *P. marinus* meronts, no increased lysozyme activity was noted compared to the non-challenged hemocytes. A methodology was developed and established to isolate and partially purify lysozyme from oyster plasma. The partially purified plasma lysozyme was further analyzed using SDS-PAGE. Two bands were identified with molecular weights of 38.8 and 18.2 kDa. When *P. marinus* meronts were treated with the partially purified plasma lysozyme, however, no significant adverse effect (e.g., viability) was detected.

IMPACTS and/or BENEFITS:
Although there is not a clear cut about the role of lysosomal enzymes in defense against *Perkinsus marinus*, results of the project provide information for a better understanding of oyster physiology and internal defense and how the two important environmental factors, temperature and salinity affect the oyster's physiology and defense against the parasite, *P. marinus*. The successful development of a methodology to isolate and partially purify lysozyme from oyster plasma is critical for further study on elucidation and characterization the role of lysozyme in the internal defense of aquatic invertebrates and molluscan bivalves in particular. Currently, colleagues at Louisiana State University (Drs. La Peyre and Xue) are employing and modifying the lysozyme purification methodology established from this project to further purify the oyster plasma lysozyme and characterize its effects in against pathogenic bacteria and *P. marinus*. Results generated from the project have been disseminated via presentations in scientific and professional conferences and publications. Two additional manuscripts are in preparation for publication.

PROJECT PUBLICATIONS:
Volety, A. K., F-L E. Chu, and L. A. Cruz-Rodriguez. 2001. Partial purification and characterization of lysozyme-like proteins from the plasma of the eastern oyster, *Crassostrea virginica*. J. Shellfish Res. 20:558.

Chu, F.-L. E. 1999. Effects of temperature, salinity, and environmental pollutants on cellular and humoral responses in oysters (*Crassostrea virginica*). J. Shellfish Res. 18: 321.

Chu, F.-L. E., A. K. Volety, and Georgeta Constantin. 1996. Intracellular and extracellular lysosomal enzyme activities in eastern oysters (*Crassostrea virginica*). J. Shellfish Res. 15:514.

FUNDING PERIOD: 10/1/99 – 9/31/01 (extended to 5/1/02)_

PROJECT TITLE: Molecular Immune Responses of The Eastern Oyster *Crassostrea virginica* to the parasite *Perkinsus marinus*.

PRINCIPAL INVESTIGATOR: Marta Gómez-Chiarri
AFFILIATION: University of Rhode Island
CO-INVESTIGATORS and AFFILIATIONS: none

PROJECT RESULTS:

In order to elucidate the molecular responses of the oyster to parasitic infection, we used suppression subtractive hybridization to construct libraries of genes involved in the response of oysters to experimental infection by *P. marinus*. We also followed the time course of protease and antimicrobial activity in the plasma of oysters experimentally infected with this protozoan parasite. Experimental infection with *P. marinus* resulted in an increase of total protease activity at early time points after infection. This increase is accompanied by the detection of low molecular weight proteases from *P. marinus,* as well as changes in the profile of oyster metalloprotease activity in oyster plasma. Our results indicate that modulation of oyster proteases by *P. marinus* could be involved in the pathogenesis of Dermo disease. Two libraries of infection-related genes were constructed by subtracting pools of cDNA isolated from hemocytes of non-infected oysters to pools of cDNA from hemocytes of infected oysters. Differential screening analysis of 96 clones from each of the libraries confirmed the isolation of 22 non-infected - specific and 54 infected –specific clones. Approximately 17% of the differentially expressed clones from each library correspond to unique sequences. Most of these clones (about 80%) do not show similarity to other sequences in the genetic databases. The infected -specific library (genes up-regulated in the process of infection) included several clones coding for arginine kinase, a phosphotransferase involved in the utilization and storage of energy that has been proposed as a marker of disease and tissue damage. It also contained a clone coding for a fragment of histone 4. Histones, major structural components of the chromatin, have also been shown to possess antimicrobial activity. However, no antimicrobial activity was detected in the plasma of oysters experimentally infected with *P. marinus.*

IMPACTS and/or BENEFITS:

This research is one further step into elucidating the complex interactions occurring between the protozoan parasite *P marinus* and it's host, the Eastern oyster, at early time points after infection. We have developed libraries and tools that will allow the identification of a large amount of gene candidates for studies in oyster immunity and host-pathogen interactions. Tools and knowledge derived from this research could lead to the development of early clinical indicators of disease, a more precisely understanding of the mechanisms of pathogenesis of Dermo, and to the development of markers for the selection of disease resistant strains of oysters.

PROJECT PUBLICATIONS:

Muñoz Ruiz P, Vance K, Gómez-Chiarri M. Protease activity in the plasma of American oysters, *Crassostrea virginica,* experimentally infected with the protozoan parasite *Perkinsus marinus.* (submitted to J. Parasitology)

Gómez-Chiarri M, Muñoz, P, Humbyrd C, Dorrington T. Antimicrobial activity in the plasma of American oysters, *Crassostrea virginica,* experimentally infected with the protozoan parasite *Perkinsus marinus.* (in preparation).

Gómez-Chiarri M., Muñoz. P., Dorrington T., Dellaporta S. Differential gene expression in hemocytes of American oysters, *Crassostrea virginica,* in response to experimental infection with the parasite*Perkinsus marinus.* (in preparation).

Gómez-Chiarri M, Muñoz. P. Differential gene expression in Eastern oysters, *Crassostrea virginica,* experimentally infected with *Perkinsus marinus*. International Conference on Shellfish Restoration. Charleston, South Carolina, USA, 2002 (conference proceedings).

Muñoz P, Gómez-Chiarri M. Protease activity in the eastern oyster *Crassostrea virginica* after experimental infection with the protozoan parasite *Perkinsus marinus*. J Shellfish Res 21(1):376 (Proceedings of the National Shellfisheries Association Meeting 2002).

Muñoz P*, Gómez-Chiarri M. Study of the immune response of the Eastern oyster *Crassostrea virginica* to the parasite *Perkinsus marinus.* European Association of Fish Pathologists, Dublin, Ireland 2001 (conference proceedings).

Gómez-Chiarri M, Muñoz P. Molecular immune responses of the Eastern oyster to the parasite *Perkinsus marinus.* International Conference on Shellfish Restoration. Hilton Head, South Carolina, USA, 2000 (conference proceedings).

FUNDING PERIOD: 9/1/97-8/31/99
PROJECT TITLE: Comparative Pathogenesis of *P. marinus* Disease in Bivalves: Development of Prevention Strategies for *C. virginica.*

PRINCIPAL INVESTIGATOR: Robert S. Anderson, Ph.D.
AFFILIATION: UMCES, CBL
CO-INVESTIGATORS and AFFILIATIONS: none

PROJECT RESULTS:
The antimicrobial activity of sera from bivalves with different resistance to dermo disease (*Crassostrea virginica, C. gigas, Mytilus edulis,* and *Geukensia demissa*) was tested from antimicrobial activity. Test microorganisms included a laboratory-propagated *P. marinus* strain and a bacterium, *Bacillus megaterium.* The growth kinetics of *P. marinus* in the presence of bivalve sera were measured turbidometrically. Bactericidal activity was quantified by determining the percent survivorship after serum exposure by the MTS/PMS assay. Activity was compared after calculating EC50 values (ug serum protein concentration required to inhibit/kill 50% of the test microbes). Serum from Maine (dermo-free) and local (dermo-exposed) *C. virginica* had comparable, low levels of anti-*P. marinus* activity, suggesting that parasite exposure was not the sole determinant of activity. Serum from *C. gigas* had no anti-*P. marinus* activity, although this species is reportedly resistant to dermo. Both mussel species tested had many hundred-fold higher anti-P. marinus activity than *C. virginica.* Anti-*B. megaterium* activity was consistently recorded for both oyster species, as well as for *M. edulis*. Antibacterial activity was not seen in *G. demissa* serum. Hemocyte extracts from all bivalves tested showed higher specific activity than did the corresponding serum samples. Ultrafiltration of bivalve sera showed that, in addition to antimicrobial proteins, *M. edulis* has active <10 kDa anti-*P. marinus* peptides that resemble defensins. These active peptides were more difficult to demonstrate in *G. demissa* and the oysters.

IMPACTS and/or BENEFITS:
These data indicate that antimicrobial agents show species-specific patterns of expression by bivalves, are produced and secreted by hemocytes, and may partially determine resistance to infectious disease. Some of the main conclusions of this study include: a) Although *C. virginica* and *C. gigas* differ in resistance to *P. marinus*, their sera contains comparable, very low levels of anti-*P. marinus* activity. b) In *C. virginica, P. marinus* exposure/infection does not influence their low level of anti-P. marinus activity. c) Although oysters have low serum anti-*P. marinus* activity, their serum contains strong antibacterial (anti-*B. megaterium*) activity. d) Mussels have serum proteins with 100- to 600-fold greater anti-*P. marinus* activity than oysters, probably a basis for their dermo resistance. e) Hemocytes secrete antimicrobial molecules in all bivalve species examined. f) Anti-*P. marinus* activity of *M. edulis* is mediated by a <10 kDa defensin-like molecule.

PROJECT PUBLICATIONS:

Anderson, R. S. and A. E. Beaven. 2000. Antimicrobial activity in cell-free hemolymph of oysters and mussels. J. Shellfish Res. 19: 641.

Anderson, R. S. and A. E. Beaven. 2001. Antibacterial activities of oyster (*Crassostrea virginica)* and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquat. Living Resour. 14: 343-349.

Anderson, R. S. and A. E. Beaven. 2001. A comparative study of anti-*Perkinsus marinus* activity in bivalve sera. J. Shellfish Res. 20: 1011-1017.

FUNDING PERIOD: 10/01/01-9/30/03

PROJECT TITLE: Antimicrobial Peptides: Overlooked Mechanisms of Disease Resistance?

PRINCIPAL INVESTIGATOR: Robert S. Anderson, Ph.D.
AFFILIATION: UMCES, CBL
CO-INVESTIGATORS and AFFILIATIONS: none

PROJECT RESULTS:
Oyster serum has been fractionated by various methods including ultrafiltration, HPLC, and column chromatography. Several molecules with antibacterial activity were isolated and characterized. One peptide likely to play a role in host defense (CVAP-1) was shown to have a mass of 4.3 kDa and to similar to defensin molecules found in other animals. It had activity against several bacterial species, as well as vs the oyster pathogen *Perkinsus marinus*. Attempts to sequence this peptide were as yet unsuccessful, probably due to insufficient sample size. Higher MW peptides with antimicrobial activity are currently under study, and several likely candidates in the >50 kDa class have been isolated. Of particular interest is a relatively large peak obtained using a QFF anion exchange column and a step-wise elution gradient. This molecule shows significant anti-*Vibrio* and anti-*Perkinsus marinus* activities; efforts are under way to measure specific activities vs a panel of microorganisms. This peptide has the advantages of relatively high concentration, broad range of activity, and technical ease of handling. In oysters many humoral defense molecules are produced and secreted by the hemocytes; therefore, we have produced sonicated hemocyte lysates, which are being fractionated on anion exchange columns. This has resulted in isolating two peptides with anti-*Vibrio* sp. which will be structurally and functionally analyzed.

IMPACTS and/or BENEFITS:
Antimicrobial peptides from *C. virginica* serum have been isolated using ultrafiltration, reverse-phase HPLC, and/or anion exchange column chromatography. The structure and function of these molecules are under study. To date, two <10 kDa and two >50 kDa peptides have been isolated with antibacterial and/or anti-*P. marinus* activities. An additional two antibacterial peptides have been isolated from C. virginica hemocyte lysates; these are being examined for anti- *P. marinus* activity as well. The major impact of this study will be a more complete understanding of *C. virginica* defenses against *P. marinus* , and other putative oyster pathogens.

PROJECT PUBLICATIONS:

Anderson, R. S. and A. E. Beaven. 2001. Antibacterial activities of oyster (*Crassostrea virginica*) and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquat. Living Resour. 14: 343-349.

Anderson, R. S. and A. E. Beaven. 2001. A comparative study of anti-*Perkinsus marius* activity in bivalve sera. J. Shellfish Res. 20: 1011-1017.

Mountz, A. and R. S. Anderson. 2002. Purification of a novel antimicrobial peptide from the Eastern oyster (*Crassostrea virginica*). J. Shellfish Res. 21: 405-406.

FUNDING PERIOD: 1995

PROJECT TITLE: Development of Nuclear DNA Markers and Pedigreed Families for Disease Resistance and Genetic Mapping in the Eastern Oyster

PRINCIPAL INVESTIGATOR: Patrick M. Gaffney
AFFILIATION: University of Delaware
CO-INVESTIGATORS and AFFILIATIONS: Standish K. Allen, Rutgers University; James C. Pierce, Philadelphia College of Pharmacy and Science

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PROJECT RESULTS:
The bacteriophage P1 cloning system was used to construct a high-quality, high molecular weight genomic library. This is the first high molecular weight genomic library from a mollusk and should prove useful for a variety of purposes, particularly genetic mapping and isolation of genes or quantitative trait loci (QTL) affecting disease resistance in the eastern oyster.

Twenty-one single-pair matings of *C. virginica* were made. Tissues from all parents were archived. Juveniles from 14 families were raised at theRutgers Cape Shore Lab. These families have been used to construct second-generation crosses, to provide a two-generation pedigreed oyster archive for genetic mapping studies.

We developed additional nuclear DNA markers to supplement the currently available set of genetic markers.

IMPACTS and/or BENEFITS: Essential tools for genetic improvement of eastern oysters, i.e., DNA libraries, additional genetic markers, and pedigreed families were developed. These will contribute to the long-term goal of developing disease-resistant oysters for population restoration and aquaculture.

PROJECT PUBLICATIONS:

Gaffney, P. M., S. K. J. Allen, and J. Pierce. 1997. Development of nuclear DNA markers and pedigreed families for disease resistance and genetic mapping in the eastern oyster: Progress report. Journal of Shellfish Research 16:257.

Gaffney, P. M., E. A. Orbacz, and Z. Yu. 1998. Using the DCode system to identify DNA sequence variation for studies of population structure in marine organisms. Pp. 4. Bio-Rad.

Wakefield, J. R., and P. M. Gaffney. 1996. DGGE reveals additional population structure in American oyster (*Crassostrea virginica*) populations. Journal of Shellfish Research 15:513.

FUNDING PERIOD: 01/01/00-12/31/01

PROJECT TITLE: Comparative examination of biochemical correlates of disease resistance in selectively bred *Crassostrea virginica* and *Crassostrea ariakensis*

PRINCIPAL INVESTIGATOR: Dr. Gustavo Calvo, Marine Scientist Senior
AFFILIATION: Virginia Institute of Marine Science

CO-INVESTIGATORS and AFFILIATIONS:
Dr. Stephen L. Kaattari, Professor, Virginia Institute of Marine Science

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PROJECT RESULTS:
Objective Ia. Assess *Perkinsus marinus* resistance in putatively resistant and susceptible stocks of oysters. Results: The final cumulative mortality of the selected groups of *Crassostrea virginica* oysters was Delaware bay (DB) < Crossbreed (XB) = Mobjack bay (MB) < Rappahannock River (RR). Concurrent MSX infection accounted for some of the early losses in the RR and MB groups. The RR oysters died within the first year of the experiment, most likely due to low resistance to MSX. The *P. marinus* body burden of the XB oysters was higher than in the DB and MB oysters; however, this was likely due to their more rapid attainment of market size and, thus, their higher rate of filtration. XB oysters reached Virginia market size within the first year of the experiment, and well before the onset of second year mortalities. In laboratory challenge of susceptible and resistant *C. virginica* and *C. ariakensis* oysters, *P. marinus* infections were found to be lower in the XB and DB groups than in the RR or *C. ariakensis* groups, as assessed by total parasite body burden. This is in contradiction to previous findings that the prevalence and intensity of *P. marinus* infections in *C. ariakensis* deployed in the field were lower than that found in *C. virginica*. The unexpectedly elevated levels of acute *P. marinus* infections in the *C. ariakensis* oysters may have been an artifactual result of mantle cavity challenge with large numbers of *P. marinus* cells. This result may have implications as the mechanism by which oysters of differential susceptibility are able to handle and clear the steady stream of infectious cells encountered during normal feeding in contrast to the large pulses of infective cells administered during artificial infection.

Objective Ib. Assess the potential correlation between endogenous oyster plasma protease and low molecular weight protease inhibitor activities and *P. marinus* resistance. Results: The assessment of the correlation of protease and protease inhibitor activity was not possible due to the presence of parasite in all field samples, and the inability to extract adequate hemolymph from the small oysters held in quarantine.

Objective Ic. Assess the potential correlation between inducible oyster plasma protease and protease inhibitor activities and *P. marinus* resistance. Results: The oyster plasma total protein content in all oyster groups underwent a seasonal variation, with progressively lower plasma protein content as the summer progressed. The initial decrease in protein concentration coincided with the occurrence of MSX disease, and the magnitude of the decline concentration corresponded with the degree of strain susceptibility. There was no apparent correlation between the level of oyster plasma inhibitory activity against *P. marinus* proteases and the parasite body burden. The protease inhibitory activity was similar between all groups at all time points. This contradicts previous research (Oliver et al, 2000), but this difference may be due to optimization of the hide powder azure technique to render it more sensitive and decrease the variability between replicates. In laboratory challenges of susceptible and resistant *C. virginica* as well as *C. ariakensis*, there were no differences in the level of protease inhibitory activity between oysters; however, there was an apparent upregulation in the protease inhibitor activity in all oyster groups following experimental infection with *P. marinus*. While the ubiquity of this response precludes its use as a screening tool for potentially disease resistant oysters, it does open a potential avenue in the study of the oyster innate immune repertoire.

Objective IIa: Assess *P. marinus* resistance in putatively resistant and susceptible stocks of oysters by assessment of inhibition of plasma degradation by *P. marinus* protease mediated by low molecular weight constituents of

resistant oyster plasma. Results: This experiment was expanded into a large matrix, where the susceptibility of all plasma from all oyster groups to degradation by *P. marinus* proteases was monitored in the presence or absence of the low molecular weight fraction of all oyster groups. Plasma proteins from all oysters groups were susceptible to degradation by *P. marinus* proteases, as determined by the appearance of numerous protein degradation fragments and disappearance of some parent bands. This degradation was not eliminated by the low molecular weight fraction of any of the oyster strains, indicating that the ability to prevent proteolytic degradation of plasma proteins may not be crucial to the progression or final outcome of *P. marinus* infection.

Objective IIb. Determine if resistant oyster plasma is capable of digesting *P. marinus* ECP. Results: Coincubation of oyster plasma with ECP did not result in degradation of the ECP proteins. Conversely, substantial degradation of hemolymph proteins occurred. In order to track the degradation of the oyster proteins, the proteins were labeled with biotin molecules prior to co-incubation with *P. marinus* ECP. This allowed an enzyme-coupled streptavidin molecule to be used as a probe, rather than a polyclonal antiserum. This had the advantage of ensuring that all proteins were tracked, regardless of their immunogenicity, and that the degradation products of those proteins could also be tracked, even if they were no longer recognized by the antiserum. The experiment demonstrated that the degradation of certain plasma proteins coincided with the appearance of apparent degradation products, some of which were of a similar molecular weight between three different oyster species. It was noted in a separate experiment that the pattern of plasma proteins visible by SDS-PAGE is relatively simple in young, uninfected oysters prior to co-incubation with *P. marinus* ECP. Comparison of protein patterns either by one or two dimensional electrophoresis or by mass spectroscopy may provide an early indicator of infection by tracking the products of exposure to specific *P. marinus* proteases.

Objective IIc: Determine if co-incubation of Dermo resistant *Crassostrea* plasma with *P. marinus* cells inhibits their replication or viability. Results: Rather than the simple retardation of growth anticipated, oyster tissue extracts and hemolymph actually induce the production of *in vivo* type forms of the parasite (e.g. tomonts, prezoosporangia) not commonly seen in *in vitro* culture. This demonstrates that the inclusion of oyster extracts into the cell culture can be used to study the mechanisms of activation and differentiation of *P. marinus* cells. Furthermore, the inclusion of these supplements caused an upregulation of a set of low molecular weight proteases to be secreted by *P. marinus* into the cell culture supernatant. This upregulation did not occur in the presence of homogenates of *P. marinus*-tolerant oyster species. It was reasoned that this "reversion" to a form more closely resembling the *in vivo* state would be associated with an increase in virulence of the *P. marinus* cell line.

Objective IId. Determine the *in vivo* capacity of purified proteases or inhibitors to retard or protect against *P. marinus* infection. Results: Inhibition of proteolytic activity did not occur in the oyster samples, as demonstrated in objective IIa; therefore, it was considered more appropriate not to simply co-inject *P. marinus* cells with protease or potential protease inhibitors, but rather to determine if supplements with homogenates or plasma would induce a more virulent cell. Thus we used *P. marinus* cells cultured in the presence or absence of *C. virginica* hemolymph or tissue homogenate to experimentally infect oysters. The body burden at four weeks demonstrated a significant increase in the infection level in the oysters administered *P. marinus* cells grown in the presence of oyster products. These results have led to a new grant which aims to determine the mechanisms of regulation of *P. marinus* proteases and other extracellular products by exposure to oyster products, and the potential association of upregulation of these products with enhanced parasite virulence.

IMPACTS and/or BENEFITS:

The results of this grant have enhanced the understanding of the potential mechanisms of *P. marinus* differentiation and virulence in both susceptible and tolerant hosts. The availability of media supplements that enhance differentiation and alter the expression of potential protease and other virulence factors has created the ability to use subtractive techniques of immunization and hybridization to attempt isolation and characterization.

Furthermore, it has enabled the search for the molecular cues present in susceptible oysters that are responsible for this phenotypic change.

PROJECT PUBLICATIONS:

Earnhart, C. and Kaattari, S. Optimization of Protease Inhibitor Assays for Eastern Oyster (*Crassostrea virginica*) Immune Assessments. Presentation to the Eastern Fish Health Workshop, 2000.

MacIntyre, E. and Kaattari, S. Altered *Perkinsus* protease profiles upon exposure to selected oyster tissue homogenates. Presentation to the Annual Meeting of the National Shellfisheries Association, 2001, Orlando, FL.

Kaattari, S., MacIntyre, E. and Earnhart, C. Modulation of *Perkinsus marinus* functions by host-derived products. Presentation to the Annual Meeting of the National Shellfisheries Association, 2002, Mystic, CT.

MacIntyre, E., Earnhart, C. and Kaattari, S. In press. Host oyster tissue extracts modulate *in vitro* protease expression and cellular differentiation in the protozoan parasite, *Perkinsus marinus*. Parasitology

Calvo, G., Earnhart, C. and Kaattari, S. Disease resistance and potential biochemical correlates in a selectively bred oyster strain. Presentation to the Annual Meeting of the National Shellfisheries Association, 2001, Orlando, FL.

FUNDING PERIOD: 10-01-01 to 9-30-03

PROJECT TITLE: Interactions of Crassostrea virginica hemocytes with the putative etiological agent of juvenile oyster disease (JOD).

PRINCIPAL INVESTIGATOR: Boettcher, Katherine
AFFILIATION: Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469-5735

CO-INVESTIGATORS AND AFFILIATIONS: None

PROJECT RESULTS:
The goal of this research is to understand Crassostrea virginica immunocompetence against the putative agent of juvenile oyster disease. The bacterium, Roseimarina crassostreae (gen. nov., sp. nov.; Boettcher in review IJSEM), colonizes the external body tissues of juvenile oysters. Once established, the bacteria induce signs such as conchiolin deposition, emaciation, and (shortly thereafter) mortality rates that may exceed 90% of annual production. As invertebrates, oysters rely primarily on hemocyte-mediated immunity. Thus, the focus of this project is to determine the contribution of host, pathogen, and environmental factors to the process of hemocyte-mediated killing of the JOD-bacterium. This work is still in progress, and a brief summary of results to date follows:

We are developing a 96-well microplate assay (using guidelines from a previously used protocol) to evaluate the ability of hemocytes to kill target bacteria. Briefly, a tetrazolium dye compound is used as an indicator of bacterial viability after incubations of bacteria with hemocytes extracted from oysters. Respiring cells reduce the dye to produce a formazan product that has a maximal absorbance at 490nm. The intensity of absorbance is determined for suspensions of bacteria alone, hemocytes alone, and bacteria in the presence of hemocytes. A killing index (KI; the percentage of bacteria killed) is then calculated from these values to yield a quantitative measure of cellular immunocompetence.

In experiments using Vibrio parahaemolyticus as the target bacterium (an organism known to be sensitive to hemocyte killing), the following parameters have been optimized: (i) hemocyte collection and washing conditions, (ii) elimination of non-target cells in hemolymph samples, (iii) concentrations of bacterial cells and hemocytes, (ii) bacteria:hemocyte ratios, (iii) killing time and temperature, (iv) growout time and temperature, (v) formazan product development time and temperature.

Preliminary results indicate that the JOD-associated Roseobacter is less susceptible than V. parahaemolyticus to killing by oyster hemocytes.

Table 1: Susceptibility of V. parahaemolyticus and the JOD-associated Roseobacter to hemocyte-mediated killing. KI indicates relative survival of bacterial cells (mean of four replicate wells).

V.Parahemolyticus:		Roseobacter:	
Initial cell conc	KI	Initial cell conc	KI
$1x10^7$	10.17	$1x10^7$	10.87
$4x10^6$	47.79	$4x10^6$	22.08
$2x10^6$	65.98	$2x10^6$	32.77
$1x10^6$	95.96	$1x10^6$	39.80

We are also evaluating and comparing this dye-reduction assay with a plate count assay for optimal quantification and reproducibility of trials. The best method will be employed for (i) investigating the effects of temperature,

salinity, oyster age and size, and bacterial strain differences on killing of Roseimarina by C. virginica hemocytes, (ii) comparing the anti-Roseimarina activities of hemocytes taken from other species of bivalves with that of C. virginica, and (ii) directly visualizing the bacterial-hemocyte interactions via epifluorescence and electron microscopy.

IMPACTS and/or BENEFITS:
This project is not yet completed. However, a basic understanding of host/pathogen interactions is expected to lead to improved grow-out methods by oyster growers. Specifically, we will have identified host and environmental parameters that result in optimal killing of the JOD-bacteria by oyster hemocytes.

PROJECT PUBLICATIONS:

None to date- project still in progress.

FUNDING PERIOD: 1995-1998

PROJECT TITLE: AN INVESTIGATION OF THE STRESS RESPONSE SUMMER MORTALITY AND DISEASE RESISTANCE IN *CRASSOSTREA GIGAS*

PRINCIPAL INVESTIGATOR: Carolyn S. Friedman and James S.Clegg
AFFILIATION: University of California at Davis
CO-INVESTIGATORS and AFFILIATIONS: Gary N. Cherr, University of California at Davis

PROJECT RESULTS:
This study characterized, for the first time, the stress response of the Pacific oyster, *Crassostrea gigas*. This species has the strongest stress response ever determined as evidenced by the production of stress or heat-shock proteins (hsp) and development of thermal tolerance (ITT) for up to 3 weeks.

- *C. virginica has a shorter stress response (hsp production and ITT) than C. gigas.*
- We developed a heat shock protocol and western blotting methods for the assessment of hsps in oysters.
- We demonstrated that using a sub-lethal heat shock on field-planted oysters shows promise to reduce mortalities in specific field situations.
- Oysters planted at high tidal heights (i.e. 3 ft above MLLW) experienced higher losses, failed to produce hsp 69.
- We characterized the hsp production of an oyster pathogen, *Nocardia crassostreae*, upon heat shock and determined that the hsp production lasted less than 1 hr. This information was used to test the stress response of Pacific oysters exposed to this bacterium.
- Using controlled laboratory studies, exposure to nocardiosis reduced survivorship. However, the oysters were still able to mount a stress response.
- We determined that exposure to hyposalinity delays the stress response: hsp production and ITT occur later than in ambient salinity cohorts. This response was independent of modulations in protein and moisture content.
- The cellular immune response of Pacific oysters was characterized under normal and stressed conditions (with nocardiosis and/or heat shock) and observed no significant effect in immune response upon exposure to nocardiosis or nocardiosis plus heat shock ($p<0.05$). However, we did observe significant alterations in immune cell function as a result of heat shock and reproductive maturation: immune capabilities were reduced.

IMPACTS and/or BENEFITS:
This research has had a tremendous impact on oyster culturists and researchers world-wide. Due to our studies, we understand the physiology of the oyster losses enough to better manage these losses. In addition, the tools we created are being used by researchers in many areas.

PROJECT PUBLICATIONS:
Friedman, C.S., Shamseldin, A., Olin, P.G., Robbins, T.T., and Cherr, G.N. In review. Investigation of a mass mortality of the Pacific oyster, *Crassostrea gigas* Thunberg, in Tomales Bay, California. Journal of Shellfish Research.

Hamdoun A M ; Cherr G. 2001. N. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for resting thermal limits and induction of thermal tolerance. American Zoologist. 41(6):1643-1644.

Hamdoun Amro M; Cheney Daniel; Elston Ralph; McDonald Brian; Cherr Gary N. 2000. Summer stress protein responses of cultured Pacific oysters: Does chronic stress
reduce tolerance? Journal of Shellfish Research. 19(1):599.

Friedman, C.S.,Cherr, G.N., Clegg, J.S., Hamdoun, A.H., Jacobsen, J.L., Jackson, S.A., and Uhlinger, K.R. 1999. Investigation of the stress response, summer mortality and disease resistance of oysters, *Crassostrea spp*. Journal of Shellfish Research 18(1):297.

Clegg, J.S., Uhlinger, K.R., Jackson, S.A., Cherr, G.N., Rifkin, E., and Friedman, C.S. 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster, *Crassostrea gigas*. Molecular Marine Biology and Biotechnology 17(1):79-83.

Shamseldin Ally A; Clegg James S; Friedman Carolyn S; Cherr Gary N; Pillai Murali C. 1997. Induced thermotolerance in the Pacific oyster, *Crassostrea gigas*. Journal
of Shellfish Research. 16(2):487-491.

Friedman Carolyn S; Shamseldin Ally; Pillai Murali; Olin Paul G; Cherr Gary N; Jackson Susan A; Rifkin Erik; Uhlinger K R; Clegg James S. 1997. Summer mortality and the
stress response of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research. 16(1): 335.

FUNDING PERIOD: 10-1-01 to 9-30-03

PROJECT TITLE: Oyster Herpes Virus Threat to U.S. Oyster Producers

PRINCIPAL INVESTIGATOR: Dr. Ralph Elston
AFFILIATION: Pacific Shellfish Institute
CO-INVESTIGATORS and AFFILIATIONS:
Dr. Bruce Barber, University of Maine; Dr. Eugene Burreson, Virginia Institute of Marine Science; Dr. Carolyn S. Friedman, University of Washington; Dr. Kimberly Reece, Virginia Institute of Marine Science

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PROJECT RESULTS:
We have tested Eastern oyster (*Crassostrea virginica*) larvae and spat from Virginia, Louisiana and Maine using the polymerase chain reaction procedure for oyster herpes virus and all groups have tested negative. The procedure was first validated by K. Reece and N. Stokes of VIMS. Similarly, tests of Pacific oyster (*C. gigas*) spat from Washington state were negative for the herpes virus. Oysters from Tomales Bay, California tested PCR+ for the herpes virus (Fig. 1) using the first set of PCR primers ("A" primers) when examined by team member C. Friedman of the U.W. Our recent results indicate an infection prevalence ranging between 3.3% and 43% at a single site in Tomales Bay, California. Historical examinations suggest that herpes virus may not be widespread in the U.S. because we have not observed the degree of mortality or lesions described by European researchers where the disease occurs in both larval and juvenile oysters.

IMPACTS and/or BENEFITS:
The recent (October 2002) PCR+ results from Tomales Bay, California indicate that it is of critical time importance to establish the nature and magnitude of this threat to U.S. producers. As a precautionary measure, it should be assumed that this virus is not widespread in cultured stocks of Eastern or Pacific oysters in the United States and further, that U.S. oyster stocks that are not infected may be at risk should they become infected. Therefore, the continuation of the study is all the more critical in order to define its geographic distribution, evaluate its significance to U.S. fishery resources and recommend appropriate management steps which could include actions to prevent further spread of the virus. Government representatives from French and New Zealand fishery agencies have opposed the designation of this virus disease as being Notifiable by the international animal health advisory organization, the Office Internationale des Epizooties (O.I.E.). The presence or absence and relative impact of the virus variant form we have found in California that apparently is highly significant to hatcheries in France and New Zealand remains largely uncharacterized. Our results to date using the polymerase chain reaction method have failed to detect the virus from samples of both east (*C. virginca*) and most west coast (*C. gigas*) oyster larvae and juveniles, suggesting a localized distribution of this virus. Until a scientific study more thoroughly documents the absence or presence of the herpes virus in both hatchery and nursery produced stocks and adult stocks, measures cannot be taken to preclude its introduction, or its spread. Conversely, appropriate management procedures cannot be formulated until its presence and distribution are established in locations where the virus is identified.

PROJECT PUBLICATIONS:

Herpes virus in Pacific oysters from Tomales Bay, California (in preparation, C. Friedman, lead author)

FUNDING PERIOD: 12-1-97 to 11-30-99

PROJECT TITLE: Mortality of the oyster, *Crassostrea gigas*: health screening, environmental links and management options

PRINCIPAL INVESTIGATOR: Dr. Dan Cheney
AFFILIATION: Pacific Shellfish Institute
CO-INVESTIGATORS and AFFILIATIONS:
Dr. Ralph Elston, Pacific Shellfish Institute; Dr. Gary Cherr, University of California at Davis, Bodega Marine Laboratory

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PROJECT RESULTS:
Research was begun in Washington and California to characterize the Pacific oyster, *Crassostrea gigas*, summer mortality disease in a variety of culture conditions and locations, and to describe the relationship to infectious diseases, and identify water quality and seasonal patterns. Also, as a continuing study was a field component to investigate the oyster thermal stress response and an assessment of induced thermal tolerance to reduce mortalities. There were obvious differences in the disease rates, with triploid oysters having consistently higher mortality rates than diploid oysters planted in comparable plots. Mortalities trended toward higher rates at or immediately after neap tides when DO was lowest and during periods of elevated air and water temperatures. A long period of neap tides with low and slack water during the evening was observed to result in daily and successive reductions in DO to levels ranging from 0.5 and 2 mg/L. The DO reductions were sometimes coupled with heavy macroalgae blooms and high phytoplankton densities. Low daytime tides coupled with intense insolation resulted in very high ambient air temperatures and elevated water temperatures on the incoming tide. During the summer 1998, peak temperatures neared 53 C (127 F) and frequently exceeded 40 C during low-tide exposure at several of the Washington sites. Relative densities of the phytoplankton *Akashiso sanguinea*, *Ceratium* spp., and *Psuedo-nitzschia* spp. were higher during the same late summer period of elevated mortalities; however, no mortalities of larval or juvenile oysters occurred when challenged with cultured *Akashiso sanguinea*. Systemic bacterial infections in moribund oysters were observed to uniformly result from gram negative rod-shaped bacteria. These infections were believed to occur in oysters weakened by the damage to the digestive gland. Finally, field data suggested that oysters which experienced chronic summer stress, responded by acquiring some degree of thermo-tolerance, but were unable to mount a complete stress response as observed in non-stressed animals. This inability to respond to additional acute stress appeared to be correlated to increased summer mortality.

IMPACTS and/or BENEFITS:
It is likely that Pacific oysters at the west coast study sites experienced varying degrees of chronic stress due to multiple environmental factors. This work indicated oyster summer mortality rates were strongly influenced by elevated air and water temperatures, lowered dissolved oxygen levels, and oyster ploidy. An inability to respond to *additional* acute stress appears to be correlated to increased summer mortality. As such, we proposed that the stress response can be used as an "early warning" indicator of summer mortality in Pacific oysters. Our evaluation of the data from two seasons of relatively high mortalities supported earlier reports on the rate and timing of mortality events. In addition, we identified and presented management practices for commercial cultivation as possible measures to reduce the frequency and extent of oyster losses.

PROJECT PUBLICATIONS:

Clegg, J.S., Uhlinger, K.R., Jackson, S.A., Cherr, G.N., Rifkin, E., and Friedman, C.S. 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster, *Crassostrea gigas*. Molecular Marine

Biology and Biotechnology 17(1):79-83.

Friedman, C.S.,Cherr, G.N., Clegg, J.S., Hamdoun, A.H., Jacobsen, J.L., Jackson, S.A., and Uhlinger, K.R. 1999. Investigation of the stress response, summer mortality and disease resistance of oysters, *Crassostrea spp.* Journal of Shellfish Research 18(1):297.

Friedman, Carolyn S.; Shamseldin, Ally; Pillai, Murali; Olin, Paul G.; Cherr, Gary N.; Jackson, Susan A.; Rifkin, Erik; Uhlinger, K. R.; and Clegg, James S. 1997. Summer mortality and the stress response of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research. 16(1): 335. (related research, not directly supported by this project)

Shamseldin, Ally A.; Clegg, James S.; Friedman, Carolyn S.; Cherr, Gary N.; and Pillai, Murali C. 1997. Induced thermotolerance in the Pacific oyster, *Crassostrea gigas*. Journal of Shellfish Research. 16(2):487-491. (related research, not directly supported by this project)

FUNDING PERIOD: 12-1-97 to 11-30-99

PROJECT TITLE: Mortality of the oyster, *Crassostrea gigas*: health screening, environmental links and management options

PRINCIPAL INVESTIGATOR: Dr. Dan Cheney
AFFILIATION: Pacific Shellfish Institute
CO-INVESTIGATORS and AFFILIATIONS:
Dr. Ralph Elston, Pacific Shellfish Institute; Dr. Gary Cherr, University of California at Davis, Bodega Marine Laboratory

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PROJECT RESULTS:
Research was begun in Washington and California to characterize the Pacific oyster, *Crassostrea gigas*, summer mortality disease in a variety of culture conditions and locations, and to describe the relationship to infectious diseases, and identify water quality and seasonal patterns. Also, as a continuing study was a field component to investigate the oyster thermal stress response and an assessment of induced thermal tolerance to reduce mortalities. There were obvious differences in the disease rates, with triploid oysters having consistently higher mortality rates than diploid oysters planted in comparable plots. Mortalities trended toward higher rates at or immediately after neap tides when DO was lowest and during periods of elevated air and water temperatures. A long period of neap tides with low and slack water during the evening was observed to result in daily and successive reductions in DO to levels ranging from 0.5 and 2 mg/L. The DO reductions were sometimes coupled with heavy macroalgae blooms and high phytoplankton densities. Low daytime tides coupled with intense insolation resulted in very high ambient air temperatures and elevated water temperatures on the incoming tide. During the summer 1998, peak temperatures neared 53 C (127 F) and frequently exceeded 40 C during low-tide exposure at several of the Washington sites. Relative densities of the phytoplankton *Akashiso sanguinea*, *Ceratium* spp., and *Psuedo-nitzschia* spp. were higher during the same late summer period of elevated mortalities; however, no mortalities of larval or juvenile oysters occurred when challenged with cultured *Akashiso sanguinea*. Systemic bacterial infections in moribund oysters were observed to uniformly result from gram negative rod-shaped bacteria. These infections were believed to occur in oysters weakened by the damage to the digestive gland. Finally, field data suggested that oysters which experienced chronic summer stress, responded by acquiring some degree of thermo-tolerance, but were unable to mount a complete stress response as observed in non-stressed animals. This inability to respond to additional acute stress appeared to be correlated to increased summer mortality.

IMPACTS and/or BENEFITS:
It is likely that Pacific oysters at the west coast study sites experienced varying degrees of chronic stress due to multiple environmental factors. This work indicated oyster summer mortality rates were strongly influenced by elevated air and water temperatures, lowered dissolved oxygen levels, and oyster ploidy. An inability to respond to additional acute stress appears to be correlated to increased summer mortality. As such, we proposed that the stress response can be used as an "early warning" indicator of summer mortality in Pacific oysters. Our evaluation of the data from two seasons of relatively high mortalities supported earlier reports on the rate and timing of mortality events. In addition, we identified and presented management practices for commercial cultivation as possible measures to reduce the frequency and extent of oyster losses.

PROJECT PUBLICATIONS:

Clegg, J.S., Uhlinger, K.R., Jackson, S.A., Cherr, G.N., Rifkin, E., and Friedman, C.S. 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster, *Crassostrea gigas*. Molecular Marine Biology and Biotechnology 17(1):79-83.

Friedman, C.S.,Cherr, G.N., Clegg, J.S., Hamdoun, A.H., Jacobsen, J.L., Jackson, S.A., and Uhlinger, K.R. 1999. Investigation of the stress response, summer mortality and disease resistance of oysters, *Crassostrea spp.* Journal of Shellfish Research 18(1):297.

Friedman, Carolyn S.; Shamseldin, Ally; Pillai, Murali; Olin, Paul G.; Cherr, Gary N.; Jackson, Susan A.; Rifkin, Erik; Uhlinger, K. R.; and Clegg, James S. 1997. Summer mortality and the stress response of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research. 16(1): 335. (related research, not directly supported by this project)

Shamseldin, Ally A.; Clegg, James S.; Friedman, Carolyn S.; Cherr, Gary N.; and Pillai, Murali C. 1997. Induced thermotolerance in the Pacific oyster, *Crassostrea gigas*. Journal of Shellfish Research. 16(2):487-491. (related research, not directly supported by this project)

FUNDING PERIOD: 10-1-01 to 9-30-03

PROJECT TITLE: Mortality of the Pacific Oyster, *Crassostrea gigas*: identification and evaluation of multiple environmental stressors and methods to reduce associated mortalities

PRINCIPAL INVESTIGATOR: Daniel Cheney
AFFILIATION: Pacific Shellfish Institute
CO-INVESTIGATORS and AFFILIATIONS:
Ralph Elston; Pacific Shellfish Institute; Carolyn Friedman and Gary Cherr; Univ. of California, Bodega Marine Laboratory; Christopher Langdon; Oregon State Univ., Hatfield Marine Science Center; Louis Burnett, Univ. of Charleston; Jonathan Davis, Taylor Resources, Inc.

PROJECT RESULTS:
During the first project year, we examined the interaction between survivorship, growth and stress responses of differing ploidy groups from commercial hatchery stocks and family lines from the Molluscan Broodstock Program (MBP) of Oregon State University, and planting time and height, and selected environmental parameters. To examine differential performance between oyster variety and family lines and planting period, oyster families outplanted during Fall 2000 and 2001 and Spring 2001 and 2002 at 2-3 sites in California, 3 sites in Washington (Spring only), and 1 site in Oregon (Spring 2002 only) were evaluated. Fall plants survived significantly more than did oysters planted in the spring ($p<0.05$) in California. In addition, two families (one commercial strain and MBP family 10-115) outperformed MBP family 10-116 ($p<0.001$) at all locations. Triploid oysters exhibited a differential rate of mortality, versus diploids from the same hatchery stocks. Mortalities varied between growout site and were manifested primarily in sub-adult animals. During the study period inter-annual variation in phytoplankton was more pronounced than spatial variation. While suspected harmful algal species were present in low to moderate concentrations throughout the study period, phytoplankton did not appear to be directly involved in oyster mortalities during 2002. However, repeated temperature and dissolved oxygen fluctuations were observed to be associated with oyster mortalities at the Washington and California study sites.

IMPACTS and/or BENEFITS:
This project was designed to aid in assessing genetic and physiological responses of Pacific oysters to environmental factors, culture locations and culture techniques. The principal benefits include development of disease-resistant broodstock and seed, and culture management tools to reduce mortalities (e.g. farm practices and mortality predictors, animal handling protocols under high stress conditions, and identification of environmental quality issues or concerns). Because significant variations in oyster survival, growth, condition, morphology, and physiological responses were found between differing families, ploides and varieties, research was being directed to providing growers with a suite of options to reduce summer mortality disease losses. These losses can presently exceed 50% of the marketable crop. This work was coordinated with on-going ODP funded herpes, and companion USDA MBP and NMFS-funded HAB research.

PROJECT PUBLICATIONS:

Friedman, C.S., Shamseldin, A., Olin, P.G., Robbins, T.T., and Cherr, G.N. In review. Investigation of a mass mortality of the Pacific oyster, *Crassostrea gigas* Thunberg, in Tomales Bay, California. Journal of Shellfish Research.

Hamdoun, Amro M., Daniel P. Cheney, and Gary N. Cherr. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for Resting Thermal Limits and Induction of Thermal Tolerance. Biological Bulletin. In preparation.

Hamdoun A M ; Cherr G. 2001. N. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for resting thermal limits and induction of thermal tolerance. American Zoologist. 41(6):1643-1644.

WORKGROUP 4

PUBLIC HEALTH AND PROCESSING

NOTES

WORKGROUP 4

PUBLIC HEALTH AND PROCESSING

Implementation and Coordination of the Gulf Oyster Industry Program (GOIP), John Supan, 1998-2001.
The effects of freezing on *Vibrio vulnificus* in whole and half shell oysters. Doritz Mestey, T. Ballesteros and Gary E. Rodrick.

Depuration of Galveston Bay oysters (*Crassostrea virginica*) against *Vibrio vulnificus* using probiotic bacteria. Joe Fox

Development of processing procedures for oyster products to inactivate *Vibrio vulnificus* and *Vibrio parahaemolyticus* in raw oysters. P. Mallikajunan, Michael Jahncke, Susan Duncan, Daniel Farkas, Robert Grodner and Linda Andrews, September 1, 1999-March 31, 2003.

Use of bactriophage for the decontamination of oysters infected with *Vibrio vulnificus*. Donna H. Duckworth and Paul Gulig, October 1, 2000-March 31, 2002

A comparison of cryogenic freezing techniques and their usefulness in reduction of *Vibrio vulnificius* in retail oysters.Doritz Mestey and Gary E. Rodrick.

Reduction of red toxin in clams and oysters by ozone purification and relaying. Richard Pierce, Michael Henry and Gary E. Rodrick

Response of the hooked mussel *Ischadium recurvum* to relaying as a remdiation technique to reduce biofouling on oysters and documentation of its distribution in a Louisiana estuary. Earl Melancon, Badiollah Asrabadi, Dale Diaz, 1999-01 (extension to end of 2002)

Investigation of fresh oyster flavor profiles and changes following post-harvest treatments. Zhimin Xu, October 1, 2001-September 31, 2003.

Gulf oyster industry initiative — consumer attitudes and preferences for oysters. Terrill R. Hanson, Lisa O. House and Benedict Posadas, October 1999-March 2002.

Evaluating consumer attitudes and preferences toward irradiated oysters. Linda Andrews and Benedict C. Posadas, September 1999-September 2002

Technical evaluation of a freeze-heat-cool process that facilitates oyster shucking. Steve Hall and John Supan, 1998-1999.

New oyster product: Processing market research. Joanne McNeely, Gary Rodrick, Steve Otwell and David Zimet, October 1, 1999-June 30, 2001.

FUNDING PERIOD: 1998-2001

PROJECT TITLE: Implementation and Coordination of the Gulf Oyster Industry Program

PRINCIPAL INVESTIGATOR: John Supan
AFFILIATION: Office of Sea Grant Development, LSU-Baton Rouge

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In 1997, Louisiana Sea Grant assisted the Gulf Oyster Industry Council (GOIC) in developing a long-range plan to address pressing scientific issues associated with that industry. Industry leaders prevailed on Congress to fund this initiative. The National Sea Grant College Program invited Dr. John Supan to serve as coordinator for the nationally competitive program. Supan's primary responsibility was to provide liaison between industry members, oyster researchers, and the NSGO. Specific activities conducted under this mandate include:

PROJECT RESULTS:

- An initial New Orleans workshop on February 28, 1998 ascertained the state-of-the-science by nationally recognized researchers to develop research priorities affecting the economic viability of the gulf's oyster industry, identified by an Industry Advisory Panel (IAP) with representatives from each gulf state.
- Proposal solicitation (RFPs), peer-review evaluations and technical/scientific panel selections were coordinated and facilitated for FY1998-01.
- Congressional appropriations of $1 million/year over five years have funded 35 research, outreach and coordination projects addressing select priorities.
- A second workshop highlighting project results and to redefine research priorities for the 2001 RFP was held before the IAP at the World Aquaculture Society meeting in Orlando, FL on January 24, 2001.
- The program will continue, with a reauthorization this year through congressional support fostered with oyster industry support through the Louisiana Oyster Task Force, the GOIC, and the National Fisheries Institute.

IMPACTS and/or BENEFITS:

- The GOIP is an exemplary of Sea Grant programming involving industry participation in the identification of research needs, project selection, funding support and the dissemination of program results to stakeholders.
- GOIP-funded research has provided the gulf oyster industry with results addressing coastal restoration conflicts with oyster leasing, post harvest treatment scenarios, survival of hatchery-produced seed, consumer acceptance information, processing and marketing strategies, genetic advancements toward disease resistance and improved forecasting of disease-related mortality, improved coastal wastewater treatment, predation and biofouling control strategies, hydroacoustic applications to oyster leases, and initial advancements in cell line development.

PROJECT PUBLICATIONS:

Supan, J. 2000. The Gulf coast oyster industry program: An initiative to address industry's research needs. J. Shellfish Research, Vol. 19, No. 1, pp. 397-400.

FUNDING PERIOD: October 1, 1999-August 30, 2001

PROJECT TITLE: The Effects of Freezing on *Vibrio vulnificus* in Whole and Half Shell Oysters

PRINCIPAL INVESTIGATOR: Dorilz Mestey
AFFILIATION: University of Florida, Department of Food Science and Human Nutrition, Gainesville, FL
CO-INVESTIGATORS and AFFILIATIONS: T. Ballesteros and Gary E. Rodrick, University of Florida, Department of Food Science and Human Nutrition, Gainesville, FL

PROJECT RESULTS

Numerous studies have demonstrated the persistence of *Vibrio vulnificus* in oysters during the harvest, processing, and storage in warm water months. This study investigated the effects of blast, CO_2 and liquid nitrogen freezing on the *V. vulnificus* content in oysters stored at –10 F for 4 weeks. In addition, various thawing methods for the frozen oysters were investigated for the presence of *V. vulnificus*.

Two log reductions in *V. vulnificus* content were observed immediately after freezing of whole oyster or half shell oysters. Weekly analysis of *V. vulnificus* content during –10F storage of frozen whole and half shell oyster showed a steady decline in number. At 28 days, undetectable numbers were achieved for the CO_2 frozen oysters, whereas a low number of oysters frozen using liquid nitrogen or blast freezing still showed low numbers of *V. vulnificus*. When 28 day CO_2 frozen oysters were thawed by either microwave, overnight refrigeration, or force thawed with running water, *V. vulnificus* was not detected.

PROJECT PUBLICATIONS:

FUNDING PERIOD: 1999-2000

PROJECT TITLE: Depuration of Galveston Bay Oysters (*Crassostrea virginica*) against *Vibrio vulnificus* Using Probiotic Bacteria

PRINCIPAL INVESTIGATOR: Joe M. Fox
AFFILIATION: Texas A&M University
CO-INVESTIGATORS and AFFILIATIONS:

PROJECT RESULTS:

The objectives of this study were to: 1) identify species/strains of probiotic bacteria to which *V. vulnificus* is sensitive; 2) evaluate probiotic depuration of oysters artificially infected with *V. vulnificus*; 3) compare this induced depuration to that of depuration of oysters having natural or inherent colonization; 4) evaluate semi-commercial depuration of oysters using criteria developed from the previous trials; and 5) make recommendations to the industry regarding appropriate depuration guidelines. Two studies were undertaken: Study 1), laboratory-scale *in vitro* and *in vivo* studies in aquaria; and Study 2), semi-commercial scale trials in 500 L tanks. Results from Study 1 indicated good sensitivity of ATCC *Vibrio vulnificus* to Alken-Murray Clear-Flo™ 7026. Success in depuration was achieved under the following conditions: 800,000 cfu/mL density of probiotic, 48 h purging, 72 h contact with probiotic, 26 C and 22 ppt. Semi-commercial trials identified additional useful probiotic mixes, but indicated success at depuration was difficult with levels of autochthonous *V. vulnificus* in excess of 1,000 cfu/g. Semi-commercial depuration appears to be a function of both natural purging (holding) and use of probiotics. Some signs of enrichment were shown with depurant densities in excess of 800,000 cfu/mL. Depuration also appeared more successful at holding temperatures less than 26 C. Future research needs to be conducted in determining optimal environmental criteria for depuration using various probiotic mixes.

PROJECT PUBLICATIONS AND PRESENTATIONS:

Fox, J.M., Williams, K.R., Mott, J.B., and Samocha, T.M., 2001. Depuration of Galveston Bay Oysters Crassostrea virginica against Vibrio vulnificus using probiotic bacteria: Progress Report to National Sea Grant College Program. World Aquaculture 2001, January 21-25, Lake Buena Vista, Florida.

Williams, K.R., Fox, J.M., and Mott, J.B., 2001. Preliminary evaluation of the depuration of Galveston Bay oysters, Crassostrea virginica, against Vibrio vulnificus using probiotic bacteria: inherent variability of autochthonous levels. World Aquaculture 2001, January 21-25, Lake Buena Vista, Florida (poster).

Williams, K.R., Fox, J.M., Mott, J.B., and Samocha, T.M., 2001. Depuration of Galveston Bay Oysters Crassostrea virginica against Vibrio vulnificus using probiotic bacteria: a preliminary investigation. World Aquaculture 2002, January 27-30, Town and Country Resort, San Diego, California.

Bonnot, C.S., Fox, J.M., Mott, J.B., Samocha, T.M., and McKee, D.A., 2001. Evaluation of the depuration of Galveston Bay oysters, Crassostrea virginica, against Vibrio vulnificus under semi-commercial conditions using probiotic bacteria. World Aquaculture 2002, January 27-30, Town and Country Resort, San Diego.

FUNDING PERIOD: 10/1/99-9/30/01

TITLE: "ELIMINATION OF *VIBRIO VULNIFICUS* IN TRIPLOID EASTERN OYSTERS"

PRINCIPAL INVESTIGATOR: Jerome F. La Peyre
AFFILIATION: Department of Veterinary Science, Louisiana State University
CO-INVESTIGATORS and AFFILIATIONS: Richard K. Cooper, Dept. of Veterinary Science, La. State University; Terrence R. Tiersch, School of Forestry, Wildlife and Fisheries, La. State University

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PROJECT RESULTS:
Advantages of triploidy in oysters include greater growth rate and better meat quality. It has also been postulated that triploid oysters have better host defenses; energy allocated to reproduction in diploid oysters may be allocated to host defenses in triploid oysters which have impaired gonad development. The extended spawning season of Gulf coast eastern oysters and the occurrence of the human pathogen *V. vulnificus* and the oyster pathogen *P. marinus* make triploidy attractive for this region. Our objectives were to 1) compare tissue abundance of *V. vulnificus*, total bacteria and *P. marinus* between triploid and diploid oysters during two consecutive spawning seasons and 2) evaluate the elimination of *V. vulnificus* in triploid and diploid oysters in conditions mimicking relaying in offshore waters devoid of *V. vulnificus*. Offshore relaying has been shown to significantly reduce *V. vulnificus* density in oysters.

A batch of oysters containing about 50 % triploid oysters was obtained from Dr. Supan and grown off-bottom at the Grand Isle Oyster Hatchery, La. The oysters were sampled monthly from July through September 2000 and 2001, and divided into three groups. The first group of 50 oysters was placed on ice, transported to Louisiana State University (LSU) and processed within 24 h of collection. The second group of 50 oysters was transported to LSU and placed in a recirculating water system equipped with high capacity ultraviolet sterilizers for one week at 25° C. Group 1 and 2 oysters were homogenized individually and the number of colony forming units of total bacteria and *V. vulnificus*, and *P. marinus* infection intensity were determined using standard procedures. A third group of 10 oysters was processed for histological evaluation of gonad development. The hearts of all oysters were collected and used to determine ploidy by flow-cytometry. Data was analyzed using the two-factor ANOVA ($p<0.05$) followed by the least square multiple comparison of means when significant differences were found. Pearson correlation were calculated between *V. vulnificus* density, total bacteria density, *P. marinus* intensity and oyster condition index.

No significant differences in *V. vulnificus* density, total bacteria density, *P. marinus* infection intensity, and condition index were found between diploid and triploid oysters in either group 1 or 2. Only *V. vulnificus* density was significantly lower in experimentally relayed triploid oysters (group 2) than in triploid oysters processed within 24 h of collection (group 1). Although the stages of gonadal development in triploid oysters were significantly lower than diploid oysters during the two spawning seasons (group 3), triploidy was not associated with lowered *V. vulnificus* density, total bacteria density, *P. marinus* infection intensity or condition index. The lack of significant difference in condition index between diploid and triploid oysters was unexpected and may indicate that the oysters were particularly stressed in the summer of 2000 and 2001; Both 2000 and 2001 summers were characterized by exceptionally high water temperature and salinity, and extreme oyster mortality. No antibacterial activities of hemocytes and tissue homogenates against *V. vulnificus* were found between diploid and triploid oysters.

IMPACTS AND/OR BENEFITS
Triploidy was not associated with lowered *V. vulnificus* density, total bacteria density, *P. marinus* infection intensity or condition index. Our results were obtained at a time of unusually high water temperature and salinity and may not reflect results obtained under environmental conditions more typical of the Louisiana Gulf Coast.

PROJECT PUBLICATIONS:

La Peyre, J.F., Cooper, R.K., Supan, J.E. and Volety, A.K. 1999. Total bacteria and *Vibrio vulnificus* load in diploid and triploid eastern oysters in Louisiana. Journal of Shellfish Research 18:324.

La Peyre, J.F. and Volety A.K.. 2000. Effect of *Perkinsus marinus* infection on *Vibrio vulnificus* numbers in eastern oysters and hemocyte killing of *Vibrio* spp. 3rd International Conference on Molluscan Shellfish Safety. Southhampton, NY, June 19-23. Book of abstracts p. 45.

La Peyre, J.F., Nguyen, K.-L.T. and Cooper, R.K. 2000. Potential use of synthetic antimicrobial peptides against *Vibrio vulnificus* in eastern oysters. 3rd International Conference on Molluscan Shellfish Safety. Southhampton, NY, June 19-23. Book of abstracts p. 44.

Nguyen, K.-L.T., La Peyre, J.F., Supan, J.E., Tiersch, T.R. and Cooper, R.K. 2002. Total Bacteria and *Vibrio vulnificus* densities and *Perkinsus marinus* infection intensity in diploid and triploid eastern oysters (Crassostrea virginica) in Louisiana. 4th International Conference on Molluscan Shellfish Safety. Santiago de Compostela, Galicia, Spain, June 4-8.

FUNDING PERIOD: 9/1/99-3/31/03

PROJECT TITLE: Development of Processing Procedures for Oyster Products to Inactive *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Raw Oysters

PRINCIPAL INVESTIGATOR: P. Mallikarjunan
AFFILIATION: Virginia Polytechnic Institute and State University
CO-INVESTIGATORS and AFFILIATIONS: Dr. Michael Jahncke, Associated Professor and Director, Virginia Seafood Agricultural Research and Extension Center; Dr. Susan Duncan, Associate Professor, Dept. of Food Science and Technology, VPI and SU; Dr. Daniel E. Farkas, Professor Emeritus, Dept. of Food Science and Technology, Oregon Sate University; Dr. Robert Grodner, Professor Emeritus, Dept. of Food Science, Louisiana State University; Dr. Linda Andrews, Assistant professor, Dept. of food Science, Mississippi State University

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PROJECT RESULTS:
Although there are many studies carried on the thermal properties of various meat and seafood products, there were limited data available on thermal properties for oysters, especially related to the temperature. The thermal properties including thermal conductivity, specific heat, and thermal diffusivity of shucked oysters over the temperature range of 0 °C and 55 °C were determined. In addition mathematical models to describe the microwave heating of oysters were developed so that suitable microwave treatment procedures can be identified.

Pure cultures of *Vibrio parahaemolyticus* (clinical strains 03:K6) and *Vibrio vulnificus* cultures were incubated overnight in phosphate buffered saline (PBS) to obtain initial concentrations of approximately 10^8 CFU/ml. Viable plate counts of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in pure culture were determined on tryptic soy agar plates after HHP treatments of 30, 35, 45, 50, 55 and 85 k psi for 0 to 20 min. Whole eastern oysters (*Crassostrea virginica*) were cultured in an aquarium by feeding high concentration *Vibrios* culture overnight to obtain the initial microbial count of 10^6 CFU/ml inside the oyster tissues. They were also treated with high pressure of 40, 45, 50, 55 and 85 k psi. The results indicated that pure cultures of *Vibrio parahaemolyticus* were more resistant compared with *Vibrio vulnificus* at all pressure levels and at all times. . Oyster tissues provided a good shelter for bacteria survival as a five log reduction of *Vibrio parahaemolyticus* was observed at 45 k psi after 3 min high pressure treatment.

Live oysters, with naturally incurred and artificially inoculated *Vibrios,* were exposed to 0 to3 kGy dose Cobalt-60 gamma radiation. One kGy dose reduced the *Vibrios* to nondetectable levels and at the same time maintained good sensory quality, a normal shelf life of 15 days, and minimum mortality. Higher irradiation doses increased the mortality rate and reduced shelf life.

IMPACTS and/or BENEFITS:
Measurement of thermal and electric properties of oysters has filled the void in the basic physical properties related to oysters. They can be used in modeling temperature distribution inside the oyster tissues during thermal processing including microwave pasteurization. Modeling of heating profile of oyster samples provided a key to control the non-uniform temperature distribution within the sample and microwave oven for seeking optimum heating procedures. High pressure trials on *Vibrios* pure culture as well as inoculated oyster tissues have found the basic knowledge on the effectiveness of high hydrostatic pressure on oysters in shell, in addition, it made us understand more about the high pressure inactivation kinetics. Upon approval by the USFDA, irradiation processing of live oysters will provide an effective post harvest treatment for reducing the risk of *Vibrio* illnesses.

PROJECT PUBLICATIONS:

X. Hu and P. Mallikarjunan, 2001. Thermal and dielectric properties of shucked oysters. In Seventh Conference of Food Engineering (P. Mallikarjunan and G. V. Barbosa-Canovas, Editors). A proceedings of 7th Conference of Food Engineering held at Reno, NV Nov 5-9. American Institute of Chemical Engineers, New York, NY. p 356-362.

X. Hu, J. Koo, P. Mallikarjunan and M. L. Jahncke, 2002. High pressure inactivation kinetics of Vibrio vulnificus and Vibrio parahaemolytics in buffer solution and whole oysters. To be presented at the annual meeting of Institute of Food Technologists. June 15-19, Anaheim, CA.

J. Koo, X. Hu, M. L. Jahncke, and P. Mallikarjunan. 2002. Effect of High Hydrostatic Pressure on Vibrio parahaemolyticus and Vibrio vulnificus in Pure Cultures and Whole Eastern Oysters (Crassostrea virginica). To be presented at the annual meeting of Institute of Food Technologists. June 15-19, Anaheim, CA.

X. Hu and P. Mallikarjunan. 2002. Heat Transfer During Microwave Processing of Fish Gel. To be presented at the annual meeting of Institute of Food Technologists. June 15-19, Anaheim, CA.

L. S. Andrews, M. L. Jahncke, P. Mallikarjunan, and C. D. Veal, 2002. Gamma Irradiation Processing to Reduce the Risk of Vibrio Infections from Raw Oysters. To be presented at the annual meeting of Institute of Food Technologists. June 15-19, Anaheim, CA.

X. Hu and P. Mallikarjunan. 2002. Mathematical Modeling of Microwave Heating of Fish Gel using Finite Element Method. To be presented at the annual meeting of American Society of Agricultural Engineers. July 28-31, Chicago, IL.

FUNDING PERIOD: 10/1/00 – 3/31/02

PROJECT TITLE: Use of Bacteriophage for the Decontamination of Oysters Infected with Vibrio vulnificus

PRINCIPAL INVESTIGATOR: Donna H. Duckworth, Ph.D
AFFILIATION: University of Florida, College of Medicine, Gainesville, Fl. 32610
CO-INVESTIGATORS and AFFILIATIONS: Paul Gulig, Ph.D., University of Florida, College of Medicine, Gainesville, Fl. 32610

PROJECT RESULTS:

The project was designed to make raw oysters safer for human consumption by killing, with bacteriophage, the Vibrio vulnificus that infects them. We proposed to also test to see whether the bacteriophage could prevent the human V. vulnificus disease in an animal model. To do this, we had to isolate and characterize phage, study the phage's killing ability and test the killing of bacteria in experimentally infected oysters and mice. We have isolated or obtained from other workers and purified over 20 different phages and we are studying their ability to kill 57 strains of V. vulnificus. We have found that some of the phage will only kill the bacteria when sea water is present, but that many times, this sea water requirement can be met with Mg^{++} and Ca^{++}. We are working on the best ways to grow and store the different types of vulnificus phages. The work with the mice was highly successful. Iron-dextran-treated mice were injected subcutaneously with 10 times a lethal dose of V. vulnificus and injected intravenously, either simultaneously or at various times after infection, with a phage specific for the infecting bacteria. Treatment of mice with phages prevented virtually any sign of disease in the treated mice. The phage could prevent death, systemic disease (as measured by numbers of bacteria/gram of liver) and local disease (as measured by numbers of bacteria/gram of skin and also histopathological analysis). Two different phages were effective against three different V. vulnificus strains, while a third phage that required seawater to kill the bacteria was ineffective in the mice.

Work with the oysters has been slow because of the difficulty in experimentally infecting the oysters with specifically marked V. vulnificus strains. With continuing Sea Grant funds we are presently studying ways to do this and also are testing to see if we can devise conditions under which the oysters will take up specific phage. If these latter experiments are successful we will try decontaminating naturally infected oysters with a "cocktail" of phages designed to kill a variety of different strains of V. vulnificus.

IMPACTS and/or BENEFITS:

We have proven that a variety of different phages can act to kill various strains of Vibrio vulnificus both *in vitro* and in mice , raising the possibility that phage could be used both in oysters to make them safer and also as an alternative treatment in individuals infected with Vibrio vulnificus.

PROJECT PUBLICATIONS:

Cerveny, K.E., A. DePaola, D.H. Duckworth, and P.A. Gulig. 2002. Infection and Immunity 70, 6251-6262.

Cerveny, K.E. Master's Thesis submitted to the University of Florida, College of Medicine, 2000.

FUNDING PERIOD: October 1, 1998-August 31, 2001

PROJECT TITLE: Reduction of Red Tide Toxin in Clams and Oysters by Ozone Purification and Relaying

PRINCIPAL INVESTIGATOR: Richard Pierce
AFFILIATION: Mote Marine Laboratory, 1600 City Island Park, Sarasota, FL; and University of Florida, Department of Food Science and Human Nutrition, Gainesville, FL
CO-INVESTIGATORS and AFFILIATIONS: Michael Henry, Mote Marine Laboratory, 1600 City Island Park, Sarasota, FL.; and Gary E. Rodrick, University of Florida, Department of Food Science and Human Nutrition, Gainesville, FL

PROJECT RESULTS:
A study of the accumulation and purification of harmful algal biotoxins in the clam *Mericenaria mericenaria* and the Eastern oyster *Crassostrea virginica* was initiated to investigate the use of ozone as a means to enhance the natural purification of toxins. Clams were exposed to viable cell of the Florida red tide organism, *Gymnodinium breve* (5×10^6 cells/clam/day) for 9 days. Following exposure, the clams were divided into groups of natural relay vs. ozone purification. The concentration of brevetoxins (PbTx-2 and PbTx-3) in the exposure water and clam tissue was monitored by HPLC-UV analysis and by receptor-binding assay. The amount of total PbTx-2 and PbTx-3 in the original culture prior to dilution for clam exposure and brevetoxin concentration in the effluent water after exposure clams show that the clams were exposed to appropriate levels during the study. These results show the presence of brevetoxins in the spiked samples and a definite accumulation of active toxins after 10 days of exposure. After 3 days of ozone purification, the treated clams exhibited a 50% drip in the brevetoxin activity level, whereas the non-ozone treated clams exhibited 38% drop in the 10 day exposure level. Relaying the red tide contaminated clams to clean seawater for 15 days removed (100%) toxin activity when compared to non-relayed controls.

IMPACTS and/or BENEFITS:
This research is responsible for the development of viable methods (ozone depuration and relaying to clean waters) to assist the both clam and oyster farmers during a red tide outbreak. This research has demonstrated that relaying oysters and clams to clean seawater areas for 14 days resulted in the reduction of red tide toxin to below acceptable market and regulatory levels in the clam and oysters meats.

PROJECT PUBLICATIONS:
Richard Pierce, Michael Henry, and Gary Rodrick. Reduction of Red Tide Toxin in Clams and Oysters by Ozone Purification and Relaying. Published in the Proceedings of the International Molluscan Shellfish Safety Conference.

FUNDING PERIOD: 1999-01(extension to end of 2002)

PROJECT TITLE: Response of the Hooked Mussel, Ischadium Recurvum, to Relaying as a Remediation Technique to Reduce Biofouling on Oysters and Documentation of its Distribution in a Louisiana Estuary

PRINCIPAL INVESTIGATOR: Earl J. Melancon, Jr.
AFFILIATION: Biology Department, Nicholls State University, Thibodaux, LA 70310

CO-INVESTIGATORSand AFFILIATIONS: Badiollah Asrabadi, Nicholls State University; Dale Diaz, Mississippi Department of Marine Resources.

PROJECT RESULTS:
Results indicate the following: (1) An abrupt change from a low salinity to a high salinity environment by transplanting to a new site is of minimal physiological stress to mussels, and therefore of minimal influence on their removal from oysters, (2) Predation is the driving force on the removal of mussels from oysters. Predators, such as blue crabs, oyster drills, and fish (primarily sheepshead and black drum), dominated in this study. Predator abundance is predominantly a function of salinity, with higher salinity environments generally having more predators, (3) The physical process of harvesting with a dredge, pushing oysters to the rear of the deck using water cannons, and pushing oyster overboard with water cannons at the new transplant site, resulted in 33-38% of the mussels being crushed or dislodged from the oysters; The dead and dying mussels attracted predators, (4) In two June-July commercial-scale transplanting operations, one to a mid-bay (moderate-salinity) site and the other to a down-bay (high-salinity) site, predators had effectively removed enough mussels for re-harvest within five to eight days after bedding, (5) Evaporative cooling and the use of water cannon kept deck temperatures within the tolerance range for both species; summer transplanting did not reduce oyster meat yield, (6) Mussels are more abundant on oysters in low-salinity (up-bay) environments because of reduced predator abundance, (7) Mussels have a lower salinity tolerance than oysters; Monitoring oyster reefs for 20-months indicated that mussels can survive an oyster-killing freshet during summer water temperatures with salinities as low as 1ppt, and (8) Mussels were spawning and their larvae were setting between April and October.

IMPACTS and/or BENEFITS:
To improve relaying success, the following is recommended to commercial oystermen: Planting to mid-bay and down-bay habitats will remove mussels within a short period of time if predators are present; Perhaps in as little as a week during summer water temperatures. At a mid-bay transplant site, where salinities are moderate, a simple check with crab fishermen may be beneficial to determine if blue crabs are in abundance, the primary mussel predator in this study. At a down-bay transplant site where higher salinities prevail, many species of predators are usually abundant, except perhaps during a wet period. A high-salinity (down bay) transplant site may require a quicker re-harvest time than an up-bay site because of oyster predator abundance. Transplant operations that use water cannon suppress temperatures within the pile of oysters and mussels on deck and thereby allowing transport during summer months without harming oysters. Additionally, keeping a lease cultivated by breaking up oyster clusters reduces mussel fouling in mid-bay and down-bay sites. Throughout the study it was evident that in mid-to-high salinity subtidal environments the only mussels that survived were associated with clustered (bunchy) oysters that provided shelter in the crevasses. At up-bay (low-salinity) sites, cultivation and breaking of oyster clusters was not a significant deterrent to mussel fouling.

PROJECT PUBLICATIONS:

No referred journal publications have been generated by this research; Publications are in preparation for submittal. Four (4) presentations at national and regional conference.

FUNDING PERIOD: 10/01/01-09/31/03

PROJECT TITLE: Investigation of Fresh Oyster Flavor Profiles and Changes Following Post-Harvest Treatments

PRINCIPAL INVESTIGATOR: Zhimin Xu
AFFILIATION: Dept. of Food Science, LSU Agricultural Center
CO-INVESTIGATORS and AFFILIATIONS:

PROJECT RESULTS:

1) Changes of fresh oyster fatty acids during storage following post harvest treatments

Because lipid oxidation and flavor alteration are closely related to changes of fatty acids, the changes of fatty acids in oysters treated by warm-water chilled water (WWCW) and High Hydro-Pressure (HHP) pasteurization methods were investigated. The total of fatty acids in untreated and the two treated groups decreases significantly during the storage. The polyunsaturated fatty acids (PUFA) were more vulnerable than other fatty acids. The decrease of C16:1, C20:3, C20:4, and C22:6 in WWCW group were significantly lower than either untreated or HPP group. No difference was found for C18:1 and C18:2 among the three groups. The reduction of C16:1, C20:3, and C22:6 in HHP group were significantly higher than in untreated group, except, C20:4. The results indicate that HHP pasteurization method may accelerate lipid oxidation of oyster.

2) Analysis of changes of fresh oyster flavor compounds during storage

A solid-phase microextraction (SPME) method was developed to extract flavor compounds of oyster. GC-MS (Gas Chromatography – Mass Spectrometer) technique was used to identify and quantify the flavor compounds in the extract. The significantly increased volatile compounds during storage were identified as dimethylamine and dimethyl sulfide that are produced from amino acids degradation and hexanal, hexenal, 2-nonenal, 1-octen-3-ol, 1-nonen-3-ol, 2,4-octadienal, and 3,5-octadien-2-one that are produced from lipid oxidation. The advantages of the SPME method with GC-MS analysis are very simple and reliable. It could be applied to monitor the freshness of oyster.

3) Aroma characters of the flavor compounds produced from amino acids degradation and lipid oxidation

The aroma characters of the increased volatile compounds were determined using GC-olfactory method. The odor of dimethylamine and dimethyl sulfide are described as fishy and sulfur smell, respectively. The odor of lipid oxidation compounds, hexanal, hexenal, 2-nonenal, 1-octen-3-ol, 1-nonen-3-ol, 2,4-octadienal, and 3,5-octadien-2-one are described as grass, mushroom, carrot, and watermelon smells. The changes of the volatile compounds may be directly responsible to the oyster flavor alternation during storage.

IMPACTS and/or BENEFITS:

The results of this study will help the oyster industry develop more efficient safety treatments while maintaining original flavors and extending shelf life. Furthermore, the protocols for flavor assessment used in this study could be applied in the oyster quality control.

PROJECT PUBLICATIONS:

*The manuscripts for the project publications are being prepared.

FUNDING PERIOD: Oct. 1, 1999 - June 30, 2001

PROJECT TITLE: Development of Commercial Oyster Marinades for Vibrio vulnificus Control in Gulf Coast Oysters

PRINCIPAL INVESTIGATOR: Dr. Marilyn B. Kilgen
AFFILIATION: Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310
CO-INVESTIGATORS and AFFILIATIONS: Chef Kenneth Perry, Assistant Professor, and Chef Carol Gunter, Lecturer, Chef John Folse Culinary Institute, Nicholls State University

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PROJECT RESULTS:
Six prototype commercial acid marinades (pH 4) that would appeal to a wide range of regional United States ethnic taste preferences and capable of reducing the ambient levels of *Vibrio vulnificus* to non-detectable (<3 MPN/g) in gulf oysters within 24 hours at 35°C were developed in the Chef John Folse Culinary Institute at Nicholls State University (NSU). The six prototype marinades were Pacific Rim, Hawaiian, South Louisiana, Caribbean, Midwest, and Southwest. These six prototype marinades were evaluated for flavor and textural changes in sensory studies using NSU staff, faculty and students following the guidelines of the NSU Human Subjects' Institutional Review Board. The marinades were seasonally evaluated (summer (August) - fall (November) - winter (February) - spring (April) for microbiological effectiveness in reduction of *V. vulnificus* to non-detectable limits in the microbiology laboratory at NSU. Sensory evaluation at NSU during the summer and fall sensory evaluations selected South Louisiana, Caribbean, Southwest and Midwest ethnic marinades as the top four for flavor preference. The Louisiana Oyster Task Force sponsored "The Ship's Galley Featuring a Taste of Louisiana" at the Annual Boat Show in the Louisiana Superdome. A total of 1,399 individuals sampled the South Louisiana marinade at this event for a central location testing. Of these 1,116 rated the marinade as "Excellent" in flavor ratings of 1-10 to delineate "undesirable," "average," or "excellent."

All six marinades were tested microbiologically in the summer (August) and fall (November) evaluations. After selection of the top four marinades, only those were used in the winter (February) and spring (April) microbiological and sensory evaluations. The base of the six marinades was originally vinegar. This base was used in the summer and fall trials. The base was changed to lemon juice in the winter and spring trials. Acid marination in all six prototypes at pH 4.0 or less (vinegar base) at 35°C reduced levels of ambient *V. vulnificus* from 240,000 MPN/g in the summer trial to non-detectable (<0.3 MPN/g) in 24 hrs. (The U.S. Food and Drug Administration defines "non-detectable" levels of V. vulnificus as <3 MPN/g). Samples were evaluated microbiologically at 6, 12, 18 and 24 hrs. post marination. In the fall trial the vinegar base formula for all six marinades reduced level of ambient *V. vulnificus* (only 200 MPN/g) to non-detectable by 18 hours. In the winter samples, the formulas were changed to a lemon juice base and only the four marinades selected by sensory evaluation in the first two seasons were evaluated. Since the levels of*V. vulnificus* in the control oysters was <0.3 MPN/g the microbiological analysis, and the 6, 12, 18 and 24 hr. analyses were all <0.3 MPN/g *V. vulnificus* in all four ethnic marinades. In the spring (April) sample, the ambient *V. vulnificus* levels were >24,000 MPN/g in the control oysters. At 24 hrs. the South Louisiana, Midwest, and Southwest showed non-detectable levels. However, the Caribbean marinade had 75 MPN/g *V vulnificus* after 24 hrs at 35°C. The results indicated that citric acid in the lemon juice was not as effective as the acetic acid of the vinegar.

IMPACTS and/or BENEFITS:
The intended benefactors of this new oyster product development will be the Louisiana oyster industry and related industries such as restaurants, caterers, and oyster consumers. Restaurants and caterers could offer the product as an appetizer. Consumers could benefit because of increased confidence in a value-added, safety-enhanced product.

No comprehensive studies utilizing low acid marination of raw oysters for internal reduction of naturally occurring *V. vulnificus* have been conducted previously. The development of a value-added product that reduces *V. vulnificus* to non-detectable (<3 MPN/g) in raw Louisiana or Gulf coast oysters will greatly enhance efforts to regain markets and consumer confidence in gulf oysters. It could result in a significant economic benefit to the State of Louisiana and the entire Gulf coast oyster industry.

PROJECT PUBLICATIONS:

There are no publications currently from this project, but an invited presentation was made to the annual meeting of the Louisiana Oyster Industry Convention in New Orleans, LA.

Kilgen, M.B., K. Perry and C. Gunter. 2001. Evaluation of Acid Marinades for *Vibrio vulnificus* Control in Oysters. Presented to the annual meeting of the Louisiana Oyster Industry Convention. March 24, 2001. New Orleans, LA

FUNDING PERIOD: Oct 1999 - March 2002
PROJECT TITLE: Gulf Oyster Industry Initiative - Consumer Attitudes and Preferences for Oysters
PRINCIPAL INVESTIGATOR: Dr. Terrill R. Hanson
AFFILIATION: Dept. of Agricultural Economics, Mississippi State University
CO-INVESTIGATORS and AFFILIATIONS: Dr. Lisa O. House, University of Florida; and Dr. Benedict Posadas, Mississippi State University/Coastal Research and Extension Center

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PROJECT RESULTS:
Results have helped identify characteristics about oyster consumers and non-consumers that can by used to develop marketing segments and better understand consumer attitudes towards oysters. Of a survey sample of 1,376 respondents to a nationwide mail survey on seafood consumption, 43% consumed oysters at least occasionally. The average oyster consumer indicated they ate oysters 2.6 times per month.

Results of this econometric study (House, Hanson and Sureshwaran) indicate that there are statistically significant differences between the reasons why people choose to eat oysters and the reasons oyster consumers choose how often to eat oysters. One method to increase sales is targeting existing oyster consumers (market penetration) to increase their consumption of oysters. Reasons for eating oysters included consumer enjoyment of the flavor (80% of consumers) and to add variety to the diet (37%). Oyster consumers identified the main reasons for not consuming oysters more often as price (38% of consumers indicated this was a reason for not consuming more frequently), product safety (29%), and that fresh product was not available (20%). It is likely both people indicating product safety and lack of fresh product are concerned about the safety of the product. Product safety appears to be an issue where the oyster industry can continue to improve their image among oyster consumers. Approximately 44% of oyster consumers rated oyster the least safe of all seafood products when given the choice of 4 shellfish and 8 finfish products.

Consumer preference for accepting processes such as depuration, irradiation, ozonation, or pressurization to increase consumer confidence in oysters was evaluated. Overall, 43% of oyster consumers and 54% of oyster consumers concerned about product safety indicated they would increase consumption of oysters if depuration were the method used to increase the safety of oysters. When further questioned, 635 respondents indicated whether or not they would be willing to pay for a safety treatment program. Of those, 61% preferred depuration and indicated a mean willingness to pay of $0.34 per oyster *above* the raw oyster price. This indicates the oyster industry may be able to increase the quality and perception of safety of oysters through a program of depuration, as well as charge for this process if the costs do not exceed the consumer willingness to pay.

Consumers did not indicate a preference or non-preference for farm-raised oysters, nor did they indicate having a farm-raised product as a reason to consume oysters or consume oysters less frequently. Further research could be conducted to see if consumers would perceive farm-raised oysters to be safer with certain advertising messages.

Finally, consumers were asked what would increase their consumption of oysters. Respondents who indicated price, product safety and lack of availability of fresh products were most likely to indicate factors that would increase their consumption. As expected, consumers indicated a lower price would increase their frequency of consumption, but other factors, such as government safety inspection, availability of fresh products, and company safety and quality guarantees also were indicated as factors that might increase consumption for at least 20% of consumers. Again, the importance of perception of a fresh, safe product was emphasized.

Non-consumers had different reasons for not consuming oysters, mainly taste, texture and smell, followed by product safety concerns. As flavor was the most important reason consumers ate oysters, it appears to be the biggest reason why non-consumers do not eat oysters. Although product safety is again important, it is less likely the industry would persuade non-consumers to eat oysters through the same methods as they might use to convince oyster consumers to eat more frequently. Changing non-consumer perceptions of taste, smell and texture is likely more difficult. In focus groups, non-consumers that focused on taste, texture, and smell generally

had very strong negative reactions to discussing oysters. It would appear from these results that the industry should focus expansion activities on those that currently eat oysters.

Additionally, identifying characteristics about the demographics of consumers might provide insight as to what regions of the country and types of people are most likely future oyster consumers. For instance, there were larger percentages of consumers in the Southeast Atlantic, East South Central and West South Central regions of the United States, indicating that these regions are fertile grounds for targeted advertising. Oyster consumption also increased with education and in males compared to females.

IMPACTS and/or BENEFITS:
Still getting the word out and it is difficult to measure impacts of a consumer preference study, but the results are potentially very useful to scientists and marketers.

PROJECT PUBLICATIONS:

House, L., T.R. Hanson, and S. Sureshwaran. In Review. "U.S. Consumers – Examining the Decision to Consume Oysters and Frequency of Oyster Consumption." *Journal of Shellfish Research.*

House, L., S. Sureshwaran, and T. Hanson. 2002. "Consumer Attitudes towards Seafood Safety Inspection Systems in the United States." Published in 'Paradoxes in Food Chains and Networks', Proceedings of the Fifth International Conf. on Chain and Network Management in Agribusiness and the Food Industry', J.H. Trienekens and S.W.F. Omta, editors. June: 238-249.

Hanson, T.R., L.O. House, and B. Posadas. 2002. "U.S. Consumer Perceptions and Attitudes toward Oysters." Abstract published in Aquaculture America 2002 Book of Abstracts, Jan. 27-30, San Diego, CA.

House, L., S. Sureshwaran, and T. Hanson. Submitted in 2002. "Consumer Attitudes Towards Safety Inspection Systems for Catfish." *Journal of Aquaculture Economics and Management.*
Hanson, T.R. L.O. House, and B. Posadas. 2001. "Consumer Attitudes and Preferences for Oysters, Gulf Oyster Industry Program." Abstract published in the 2001 World Aquaculture Society Meetings Book of Abstracts, Orlando, FL, January 19-25, 2001.

House. L., T. Hanson, and S. Sureshwaran. "Decision to Consume and Frequency of Oyster Consumption in the United States." Presented at the Annual Meetings of the Southern Agricultural Economics Association, Orlando, Florida, February, 2002.

Hanson, T., L. House, S. Sureshwaran, B. Posadas, A. Liu. In Review. "U.S. Consumer Opinions of Oysters: Results of a 2000-2001 Survey." Submitted as a Mississippi State University Bulletin.
An "Oyster Marketing Analysis" is being prepared for MS-AL Sea Grant as per the original RFP and objective 4.

FUNDING PERIOD: 9/99 to 9/02

PROJECT TITLE: Evaluating Consumer Attitudes and Preferences Toward Irradiated Oysters

PRINCIPAL INVESTIGATOR: Linda Andrews
AFFILIATION: Mississippi State University-Coastal Research and Extension Center
CO-INVESTIGATOR: Benedict C. Posadas, State University-Coastal Research and Extension Center

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PROJECT RESULTS:
Oysters were tolerant to irradiation processing <2.0 kGy absorbed dose. They maintained a normal shelf-life with no increase in mortality when compared with control (untreated oysters). At levels >2.0 the oysters showed increased mortality of 5-8% and at 3 kGy secreted a yellow unpleasant appearing exudate. Results of the initial irradiation response of pure broth cultures determined that V. vulnificus MO-624 (log 7/g oyster meat) was more sensitive to the irradiation exposure than the V.parahaemolyticus (log 7/g oyster meat) requiring 1.5 kGy and 2.0 kGy, respectively, to reduce each to non-detectable levels. That the V. parahaemolyticus 03:K6 was less sensitive to irradiation processing than Vibrio vulnificus was not surprising. Cook (2001) reported that the V. parahaemolyticus 03:K6 was the most resistant to processing of any of the Vibrios tested in his laboratory. Naturally incurred V.vulnificus (103/g oyster meat) was reduced to non-detectable levels with 0.75 kGy irradiation. Vibrios in control oysters and oysters exposed to very low doses of irradiation when stored under refrigeration (<40 C), V. vulnificus counts were reduced over time under these storage conditions. Pathogenic V.vulnificus (MO-624) was more resistant to irradiation processing than the naturally incurred strains and was reduced to non-detectable levels with 1 kGy absorbed dose. Note: This pathogen was also reduced over refrigerated storage. Artificially inoculated Vibrio parahaemolyticus 03:K6 TX-2103(log 4/g oyster meat) proved to be somewhat more resistant than the pathogenic V. vulnificus and required up to 1.5 kGy to reduce to non-detectable levels. The V.p were less sensitive to reduction during refrigeration than the V.vulnificus.

Two consumer panels were conducted. One in December, 2001, at the Coastal Aquaculture Unit Open House (Gulfport, MS) and the other at the Boston Seafood Show, March, 2002 (Boston, MA). There were 80 tests performed at the Open house and 66 tests performed at the Seafood Show. Consumer panelists were asked to perform a triangle difference test. Panelists were presented 3 oysters in random order. Of the three oysters, two were alike and one was different. Panelists were asked to pick the one they thought was the odd sample out of the three. Out of 146 tests conducted, 56 trials resulted in correct answers. By Chi-square statistical analysis this number was determined to be less than would be expected by random chance selection (Stone and Sidell, 1985). Therefore, there was no significant difference observed between the irradiated oysters and the control samples($p<0.001$). An expert panel within the MSU community also tasted the oysters and reported that the irradiated oysters maintained a "raw like" quality, as was commented on by the consumer panelists. There were no flavor or visual changes noted. Oysters for sensory testing were irradiated within a dose range of 1-1.5 kGy gamma rays.

Consumer attitudes and preferences toward raw oysters in general, and irradiated oysters, in particular, were evaluated from results of consumer surveys conducted through personal and telephone interviews. Seventy-five personal interviews were conducted at the MSU-Coastal Aquaculture Unit (CAU) Open House in Gulfport, Mississippi on December 6, 2001. Another survey was conducted at the MSU-Coastal Research and Extension Center (CREC) booth and exhibit among 140 participants of the 2002 International Boston Seafood Show in Boston, Massachusetts on March 12-14, 2002.

Telephone interviews of adults living in the Baltimore and Houston were conducted in June 2002. Of the eligible respondents contacted in Baltimore, 610 completed the interview and 85 refused to participate. Of the eligible respondents contacted in Houston, 606 completed the interview and 67 refused to participate. The sampling error for the both surveys was no larger than " 4.0%. Respondents were asked whether they eat raw oysters or not, and

if not, indicate the main reasons for not eating raw oysters. They were also asked about their primary food safety bacteriological concerns about raw oysters, frequency of eating raw oysters, and source of raw oysters. A series of questions was asked regarding their attitude toward radiation and irradiated oysters, interest in buying irradiated raw oysters, and willingness to pay for a dozen irradiated raw oysters if purchased in the supermarket. Respondents' characteristics including sex, marital status, age, household income, and educational attainment were also asked.

A higher proportion of the respondents interviewed personally than those interviewed by telephone reported eating raw oysters in 2001. More than 60 percent of the respondents at the Boston seafood show and Gulfport aquaculture open house ate raw oysters, while 28 percent of the respondents at the Baltimore and Houston telephone interviews reported eating raw oysters in 2001. More of the male respondents tend to consume raw oysters than female respondents. Among male respondents from Baltimore and Houston MSA's, about 40 percent stated that they ate raw oysters in 2001. A lower percentage of female respondents (20%) from the two MSA's reported eating raw oysters. Among male respondents at the Boston seafood show, 69 percent reported eating raw oysters in 2001. A lower percentage of female respondents (49%) at the seafood show indicated eating raw oysters.

IMPACTS and/or BENEFITS:
This project will benefit both the oyster consumer and the oyster industry. Consumers favorably accepted the irradiated oyster and could not differentiate an untreated from an irradiated oyster. The oyster industry will benefit from the exploration and acceptance of alternative post harvest treatments to reduce the risk of vibrio illnesses. Several Mississippi and Alabama processors have expressed interest in utilizing irradiation post harvest treatment upon approval by the US Congress.

PROJECT PUBLICATIONS:

Andrews, L.S., B. D. Posadas, M.Jahncke. 2002. Oyster irradiation: pathogenic Vibrio response and consumer difference testing. Proceeding 6th Joint Meeting, Seafood Science a& Technology Society of the Americas and Atlantic Fisheries Technology Society. Orlando October 9-11. (This is an extended abstract, not refereed journal article).

Posadas, B. and L.S. Andrews. 2002. Consumer preferences and attitudes toward irradiated oysters at the Boston International Seafood Show. Proceeding 6th Joint Meeting, Seafood Science a& Technology Society of the Americas and Atlantic Fisheries Technology Society. Orlando October 9-11. (This is an extended abstract, not refereed journal article).

Abstracts:
Andrews, L.S. and S. DeBlanc. 2002. Gamma irradiation processing to reduce the risk of vibrio infections from raw oysters. Sixty-sixth Annual Meeting Mississippi Academy of Sciences. Biloxi, MS. Feb. 2002.

Andrews, L.S., M. Jahncke, K. Mallikarjunan and C.D. Veal. 2002. Gamma irradiation processing to reduce the risk of vibrio infections from raw oysters. Seafood Technology Division, Instititute of Food Technologists. 2002 IFT Annual Meeting, Anaheim, Ca. June.

Andrews, L.S., B. D. Posadas. 2002. Oyster irradiation: pathogenic Vibrio response and consumer difference testing. Invited for presentation at the National Shellfish Association Meeting in New Orleans, April 2003.

Posadas, B. and L.S. Andrews. 2002. Consumer preferences and attitudes toward irradiated oysters at the Boston International Seafood Show. Invited for presentation at the National Shellfish Association Meeting in New Orleans, April 2003.

FUNDING PERIOD: 1998-99 (2002-03)
PROJECT TITLE: Technical Evaluation of a Freeze-Heat-Cool Process that Facilitates Oyster Shucking

PRINCIPAL INVESTIGATOR: Steven Hall
AFFILIATION: Biological & Agricultural Engineering Department-LSU
CO-INVESTIGATOR and AFFILIATION: John Supan, Office of Sea Grant Development-LSU

This project is being conducted at LSU via a revised proposal and budget due to termination of the original PI at Mississippi State University.

PROJECT RESULTS:
From these studies, it was determined that gulf oysters can be treated to cause a relaxation of the adductor muscle that facilitates the shucking process. Several of these treatments caused a full or partial release of the adductor in 75% or more of the oysters, with peak total release rates at 85-90%. Time/temperature data was obtained from the treatments that would allow for the calculation of the conduction coefficients of both the shell and the muscle. More comprehensive studies would be required to accurately and confidently determine these coefficients.

From the microbial analyses of the treated versus the raw oysters, a lower total plate count was observed during a 14-day period in the treated oysters.

Texture analysis revealed a consistent but statistically insignificant increase in the force necessary for the compression of the treated oyster meats.

Further study would allow for a more comprehensive investigation into the treatment combinations that have already shown promise. In addition, related studies which would lead to effective use of this technology and the developed methodologies in commercial applications are recommended.

IMPACTS and/or BENEFITS:

Project continuing.

PROJECT PUBLICATIONS:

Dissertation in preparation.

FUNDING PERIOD: 10/01/99 – 6/30/01

PROJECT TITLE: New Oyster Product: Processing and Market Research

PRINCIPAL INVESTIGATOR: Joanne McNeely
AFFILIATION: Bureau of Seafood and Aquaculture Marketing, Florida Department of Agriculture and Consumer Services
CO-INVESTIGATORS and AFFILIATIONS: Dr. Gary Rodrick, Institute of Food and Agricultural Science, University of Florida; Dr. Steve Otwell, Institute of Food and Agricultural Science, University of Florida; Dr. David Zimet, Institute for Food and Agricultural Science, University of Florida, North Florida Research and Education Center

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PROJECT RESULTS:
A grant from the Florida Sea Grant College Program has allowed researchers to analyze the viability of frozen oysters and the acceptability of the products by means of laboratory, consumer and industry research. The laboratory research shows that this new product, when exposed to extremely low temperatures for specific periods of time, shows no detectable signs of the bacteria *Vibrio vulnificus*. The consumer and trade research proves there is potential for growth and the need for continual education to the trade, health care professionals and at-risk consumers about this new product.

IMPACTS and/or BENEFITS:
Previous to this research, there was no data to indicate the proportion of the United States population that has consumed oysters. Nor was there a clear demographic profile of the oyster consumer.
Individuals who believe the new method of freezing oysters can reduce the bacteria levels are more likely to purchase this new product and would be willing to pay a premium price. This can increase oyster sales, reduce illnesses, and prevent deaths, thereby benefiting the oyster industry as a whole.

PROJECT PUBLICATIONS:

A final comprehensive report including graphics and charts titled after the project.
Also available on the Bureau's website: www.fl-seafood.com.

WORKGROUP 5

AQUACULTURE AND HATCHERY ISSUES

NOTES

WORKGROUP 5

AQUACULTURE AND HATCHERY ISSUES

Cooperative Regional Oyster Selective Breeding (CROSBreed) project. Standish Allen, Ximing Guo, Susan E. Ford, Gregory A. DeBrosse, Patrick M. Gaffney, Eugene Burreson, Mark Luckenbach, Kennedy Paynter and Donald Meritt, 1995-1997.

Brood Stock management of disease resistant stocks in the hatchery. Standish K. Allen, Jr., Don Meritt and Mark Luckenbach. 1997-1999.

Cooperative Regional Oyster Selective breeding (CROSBreed) project: Potential of selected stocks for restoration and extensive planting. Standish K. Allen, Jr., Eugene Burreson, Mark Luckenbach, Patrick M. Gaffney, Ximing Guo, Gregory A. DeBrosse, Kennedy Paynter and Donald Meritt, 1999-2001.

Development of tetraploid oyster broodstock for the Gulf of Mexico region. John Supan, Terry Tiersch and Standish K. Allen, Jr. 2001-2003.

The Caribbean oyster: Genetic resource for American oyster culture? John Scarpa and David Bushek, October 1, 1999-September 30, 2001.

Optimum size for planting hatchery produced oyster seed. Richard K. Wallace and David B. Rouse, 1998-2001.

Caernarvon and oyster farmers: What happened and what it means. Sharonne O'Shea, Joe F. Stevenson and James Wilkins, September 1, 1998-December 31, 1999.

Legal authority to clean up oyster beds due to pollution. September 1, 1999-August 31, 2000. Erinn Neyrey, James Wilkins, September 1, 1999-August 30, 2000.

Recent coastal restoration and oyster leaseholder conflicts have led to changes in the stuatory and regulatory landscape: What are the changes and how will they affect the industry? Erinn Neyrey, James Wilkins and Melissa Watson, October 1, 2001-September 30, 2002.

FUNDING PERIOD: 1995 - 1997

PROJECT TITLE: Cooperative Regional Oyster Selective Breeding (CROSBreed) Project

PRINCIPAL INVESTIGATOR: Standish K. Allen, Jr.
AFFILIATION: Haskin Shellfish Research Lab, Rutgers University
CO-INVESTIGATORS and AFFILIATIONS: Ximing Guo, Susan E. Ford, Gregory A. DeBrosse, HRSL, Rutgers; Patrick M. Gaffney, Center for Marine Studies, U. Del.; Eugene Burreson, Mark Luckenbach, VIMS, College of William and Mary; Kennedy Paynter, Dept. of Biology and Donald Meritt, Horn Point Environmental Lab, U. Maryland

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PROJECT RESULTS:

Transition from institutional to regional program

The origin of CROSBreed oyster strains is rooted in experiments by Dr. "Hal" Haskins of Rutgers University from the late 1950s onward. He developed a number of lines resistant to MSX-disease caused by *Haplosporidium nelsoni*. Using these lines as founder stock, new synthetic strains of disease resistant oysters were established in 1992 at Rutgers (under PI-Allen while he was there). This development gave Rutgers the opportunity to subject their MSX resistant lines to constant Dermo-disease pressure, therefore avoiding the need to start a dual resistance selection program with wild type stock. Since 1992, the synthetic lines have been on a three-year breeding cycle. Until 1995, this breeding program was a local one, supported entirely by Rutgers.

ODRP support for our regional project began in 1995 for the continued development and testing of these new synthetic lines; from that time, the strains have been known as the CROSBreed (XB) lines. There are two key differences between the original Haskin derived lines and the XB strains. First, the XB project is a well managed selection program designed for long-term maintenance of healthy brood stock with systematic exposure to both major oyster parasites on the East Coast, *H. nelsoni* and *Perkinsus marinus*. XB lines have had about 2½ generations of selection to this latter parasite. Second, the project is regional, with New Jersey, Delaware, Maryland, and Virginia participating.

That CROSBreed has truly made the transition from institutional to regional program was evident during a 1998 Spring workshop of CROSBreed participants. The project was due to end in September of 1998 with the final task of spawning the third generation of XB lines and their controls. However, ODRP suffered a funding hiatus and no RFP was issued that year. That meant that CROSBreed participants were faced with a new generation of test stocks (8 in all, deployed in replicate at three regional testing sites). All participants agreed to undertake this work for a year until a new RFP (this one) was issued in 1999. This request, in part, is for completing the field and disease testing portions of XB selective breeding for the remaining two years of our three-year breeding cycle. The project would end in 2001 with the creation of XB 4*x* progeny. Regional cooperation not only means the addition of new talent to the breeding effort but also an expanded impact for the XB lines.

Among growers – gardeners and commercial start ups alike – an awareness is building that seed is not just seed, but that there are choices out there, whether they be XB strains, natural stocks such as from Tangier Sound, or other select lines such as the DEBY strain developed and maintained by Burreson's group at VIMS. And people are starting to request what they want from hatcheries.

Some XB oysters are destined for reef programs, where juveniles grown by gardeners are planted on reef sanctuaries. XB strains have been used for the Virginia Marine Resources Commission seaside oyster growers project, and a derivative of the XB line will be used this year again. XB lines have been requested for the Wilson Bay Water Quality Initiative in Jacksonville, NC. Because requests for XB brood stock are mounting constantly, the Aquaculture Genetics and Breeding Technology Center (ABC) now maintains an inventory of XB brood stock for distribution to prospective users. In addition, XB stocks are being used in a score of other research projects, some funded (or proposed for funding) by ODRP.
During the last three year period, XB lines performed as well or better than local controls in all three testing sites: low, medium, and high salinity. Superior performance obtained in disease prevalence and often growth, depending on the line. Ironically, the last three year period was one characterized by relatively low disease

pressure, so the full benefit of XB lines are yet to be demonstrated, that is, their performance versus controls against heavy disease challenge.

We have to remember that XB lines were produced from MSX-resistant lines, and that Dermo resistance, if it indeed occurred, was only under selection for 1-2 generations. The fact the lines contracted Dermo is not surprising. It may be that "resistance' to Dermo involves resisting death at a high infection level rather than resisting infection *per se*. In any event, we speculate that if MSX were present, XB performance would be more remarkable. Other good news is that XBs were about equivalent to local controls in all sites, indicating that the stock seems genetically robust and may be useful across a number of environments.

IMPACTS and/or BENEFITS:

CROSBreed stocks have been released to commercial hatcheries and are in use by oyster restoration programs in the Chesapeake Bay, including the Oyster Recovery Partnership in Maryland and the Chesapeake Bay Foundation.

The project results are applicable to the entire range of the eastern oyster, i.e., where ever brood stock may be multiplied to production levels through aquaculture. MSX/ Dermo resistant strains could be especially valuable in the northeast where both MSX and Dermo seem to be expanding its range and where aquaculture represents the major form of oyster harvesting. The end users of the brood stock development are the oyster aquaculturists, both the hatchery and grow out industries. Already available to growers are the MSX resistant lines and excess production of the XB lines. The Dermo resistant varieties will undoubtedly spark further interest once we have clearly demonstrated that cross breeding with a naturally resistant population accelerates resistance.

The need for a strain of oysters resistant to both MSX and Dermo is critical on the East coast. Testing methods to accelerate disease resistance using *C. virginia* races is attractive because it avoids strategies that involve non-native species. The regional significance of this project is to foster oyster aquaculture by providing culturists with disease resistant strains. Oyster hatchery and grow out programs for *C. virginica* are under development in other areas of the country as well, such as Florida and Louisiana. Dermo resistance is of considerable interest in these regions. Of national significance is the demonstration of improved shellfish performance through breeding.

FUNDING PERIOD: 1997 - 1999

PROJECT TITLE: Brood Stock Management of Disease Resistant Stocks in the Hatchery

PRINCIPAL INVESTIGATOR: Standish K. Allen, Jr.
AFFILIATION: Haskin Shellfish Research Lab, Rutgers and VIMS, College of William and Mary
CO-INVESTIGATORS and AFFILIATIONS: Don Meritt, Horn Point Lab, U. Maryland; Mark Luckenbach, VIMS, College of William and Mary

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PROJECT RESULTS:
A total of five workshops were presented in 1999 in the following states: Massachusetts, Connecticut/New York, New Jersey, Virginia, and North Carolina. Workshop presenters and the topics they covered are listed below.

Standish Allen, Jr. – formerly of Rutgers University, now with the Virginia Institute of Marine Science (VIMS), Director, Aquaculture Genetics and Breeding Technology Center (ABC) – "Genetics in brood stock development" and "CROSBreed Project Report."

Greg DeBrosse – Cape Shore facilities manager, Rutgers University, Haskin Shellfish Research Laboratory – "Practical methods of shellfish breeding." DeBrosse's segment of the workshop was a hands-on demonstration of methods used in our breeding programs.

Tom Gallivan – formerly of VIMS, ABC, now Manager of seaside clam operations and alternative species development, Cherrystone Clams, Cheriton, VA – "Clam Breeding Project."

Don Meritt – University of Maryland, Horn Point Environmental Laboratory – "Developing useful monitoring programs for your shellfish business."

Don Webster – University of Maryland, Wye Research and Education Center – "Program overview" and "Feedback for the future."

Overall we reached 64 participants: 17 hatchery operators, 35 growers, and 12 others. Response to the workshops was enthusiastic, as judged by the "End of Program Evaluations" graded by participants (see Appendix 2 – Evaluation form and summary of responses." In addition, a questionnaire was distributed concerning important character traits for a shellfish breeding and selection program that is also part of Appendix 2. This latter information was more or less for us, the "breeders" to achieve some feedback from the industry about their concerns.

One of the major objectives to these workshops was to simply educate aquaculturists to the concept that domesticated stocks of oysters (and clams) are and will be increasingly available. In other words, an oyster is not an oyster, and paying attention to its origin and how it is performing relative to other stocks (this material presented in Meritt's section) is a crucial and fundamental farming protocol. Since the workshops, CROSBreed brood stock have been distributed to hatcheries in Maryland, Virginia, and New Jersey. There are no commercial hatcheries in North Carolina. Massachusetts and New York have some restriction on the import of adults from the mid-Atlantic area (including New Jersey). More telling than perhaps the issuance of brood stock has been the demand for CROSBreed seed from the VIMS and Horn Point hatcheries. These requests represent growers who have become aware of disease resistant stocks and are interested in trial plantings of seed.

Another major objective was to educate participants about the rigors of a selection program. This began with an introduction to the principles of selective breeding by Allen. It was later reinforced by a second presentation by Allen with details of how the CROSBreed lines were derived and our current selection program on them. A more hands on explanation of these rigors and what it means from a practical point in the hatchery was covered by DeBrosse who detailed the steps and procedures we take in typical breeding programs. After this demonstration, participants were pretty much willing to allow that this process best belongs in the hands of professionals at, say, Rutgers and VIMS. IN other words, it is impractical to accomplish a meaningful selection program at a commercial hatchery.
As a final point, then, Webster presented a section called "Putting the stocks to use." Webster described the dilemma facing institutions mounting selective breeding programs: How are costs of a continuing selective breeding program borne? We then introduced the notion of licensing of the stocks to the groups. It is important to

note that licensing is a standard feature of agriculture and relatively new for aquaculture, primarily because there have been no stocks with enough added value to warrant commercialization. This has changed with the advent of CROSBreed and some other strains under development. Our objective in telling participants of the licensing strategy was not to get them to "sign up" but to try to understand the principal and justification for it. Webster circulated a handout that is attached as Appendix 3 – Putting the stocks in use or Paying for improved shellfish lines.

IMPACTS and/or BENEFITS:
This project is one of the most visible manifestations of ODRP research to the industry. In fact we are taking the products of research for ODRP and experience of our breeding program to the field. Our overall agenda is to begin to create a network of producers who are knowledgeable about genetics and breeding of shellfish and cognizant of the value of improved strains.

XB lines are becoming increasingly useful to growers, practically speaking. More and more seed have been disbursed. The most have been distributed to oyster gardeners in Virginia. Some growers in Virginia are also testing CROSBreeds. The experiences of the growers themselves, as they use the strains will not only serve to help corroborate our own data but also to help evaluate overall commercial performance and provide experiential data for later improvements.

The major development stemming at least in part from this collaborative activity among Rutgers, VIMS and University of Maryland has been the establishment of the Mid-Atlantic Shellfish Genetics and Breeding Technology Consortium. The Consortium is a memorandum of understanding among these institutions as to sharing research, intellectual property, and practical programs such as the CROSBreed project. Note that these three institutions are the official test sites of the ODRP funded CROSBreed project. We have agreed to set up a licensing system for CROSBreed (and other strains under development) brood stock. The licensing fees will be administered by Rutgers' Office of Corporate Liaison and Technology Transfer. Licensing fees have initially been set at a conservative 7% of sales (although the value of the stocks is certainly much greater than that). So far there is only one commercial hatchery producing CROSBreed seed under these licensing conditions. However, this is just the beginning of what might become widespread use of these stocks. We are confident that the participants of these workshops understand the importance of value added, domesticated brood stocks and the rationale behind their licensing.

FUNDING PERIOD: 1997 - 1999

PROJECT TITLE: Brood Stock Management of Disease Resistant Stocks in the Hatchery

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FUNDING PERIOD: 10/01/99 – 09/30/01

PROJECT TITLE: The Caribbean Oyster: Genetic Resource for American Oyster Culture?

PRINCIPAL INVESTIGATOR: John Scarpa
AFFILIATION: Harbor Branch Oceanographic Institution, Inc., Ft. Pierce, FL

CO-INVESTIGATOR and AFFILIATION: David Bushek, Baruch Marine Field Lab., Georgetown, SC

PROJECT RESULTS:

The Caribbean oyster *Crassostrea rhizophorae* is a closely related species of *C. virginica*, but little is known of its susceptibility to Dermo. Therefore, pathogenicity of Dermo was compared between the two oyster species.

Objective 1. Isolate, verify, and maintain in culture the *Perkinsus spp.* present in the Caribbean oyster. This was attempted three times, but the objective was not met. *Perkinsus* assays were positive, but revealed low infection intensities that effectively eliminated our ability to successfully isolate the parasite.

Objective 2. Propagate Caribbean and American oysters and culture the progeny under conditions that maintain them Dermo-free. American and Caribbean oysters were conditioned and spawned at HBOI. Larvae and set oysters were reared under strict quarantine using 1-?m filtered, UV irradiated seawater to prevent infection by *P. marinus*. After six to eight months, oysters had grown large enough (~50mm, some were sexually mature) for experimental use and none had become infected with Dermo, providing the first demonstration that these methods can be employed to produce SPF oysters in Florida where *Perkinsus marinus* is endemic.

Objective 3. Compare Dermo tolerance, or resistance, of Dermo-free Caribbean oysters to Dermo-free American oysters by challenging them with isolates of *Perkinsus marinus*. SPF oysters were inoculated with one of two genetically distinct isolates of *P. marinus*. One isolate was originally derived from Fort Pierce, FL oysters (ATCC 50762) and the other isolate was from Cotuit Bay, MA (ATCC 50783). Only fifteen American oysters and one Caribbean oyster died during the course of the 16-week experiment. However, whole-body burden infection intensities (log10 transformed) in surviving oysters were significantly heavier for Caribbean oysters (1.25 "0.08) compared to American oysters (0.72 "0.06). Control oysters did not develop infections (0.09"0.01).

Objective 4. Compare "natural" Dermo infection levels in Caribbean oysters to American oysters. In January 2001, SPF oysters were placed in an upland system receiving unfiltered seawater. Effluent was passed through a chlorination tank and UV-system to prevent release of gametes of the non-native Caribbean oysters into local waters. A second control group of SPF American oysters was placed in the channel that provided the source of water for the tanks. Growth and survival of this second control group of oysters were much better (1303% wt gain, 89% survival) than those held in the experimental tanks (56-130% wt gain, 0-27% survival), indicating that our experimental conditions were less than optimal. Nonetheless, comparisons between the experimental populations were valid and revealing because replicates of both species were maintained together in the tanks. In the tanks, American oysters grew faster than Caribbean oysters (130% vs 56%), but Caribbean oysters had higher rates of overall survival (10% vs 2%). After 103 days, only a few parasites were detectable in about 50% of the oysters of either species. After 214 days, virtually all of the American oysters had died and prevalence of *P. marinus* was 100% in survivors; 24% of which had moderate to heavy infections (>1000 parasites/g wet meat weight). Most Caribbean oysters had died as well, although survival was better and 10% remained free of *Perkinsus*. Only 13% of surviving Caribbean oysters had moderate to heavy infections.

The results indicate that Caribbean oysters have the same or slightly less resistance to North American isolates of *Perkinsus* as compared to American oysters, but may be more tolerant of heavier parasite loads because mortality was higher in American oysters. The higher survival levels and tolerance to higher parasite burdens of the Caribbean oyster compared to the American oyster indicate that there is a genetic component needing further study, but efforts to use *C. rhizophorae* as a genetic resource for directly improving resistance of *C. virginica* to *P.*

marinus are unlikely to yield productive results. Further comparisons may prove valuable in the elucidation of mechanisms of parasite invasion, parasite virulence, and host resistance.

IMPACTS and/or BENEFITS:

Our demonstration that Dermo-free oysters can be produced in Florida, where *Perkinsus* is widespread, is very significant. First, it demonstrates that it is not necessary to rear oysters in waters where the parasite is not known to occur to obtain Dermo-free oysters. More importantly, the ability to produce and rear SPF oysters in Florida. with only slight modifications to existing hatchery technology has tremendous implications for the shellfish industry. Presently, several states have regulations against importing clams or oysters from hatcheries in Florida unless they are certified to be free of *Perkinsus*. For out of state customers, Florida hatcheries should be able to virtually guarantee a supply of Dermo-free seed using the methods demonstrated in this project.

PROJECT PUBLICATIONS:

Bushek, D., Scarpa, J. and Laramore, S.E. 2002. Susceptibility of the Caribbean oyster *Crassostrea rhizophorae* to *Perkinsus marinus*. J. Shellfish Res., 21: 371-372 (abstract from Nat. Shellfish. Assoc. 94th Conference, Mystic, CT, 14-18 April 2002).

Scarpa, J., Bushek, D. and Laramore, S.E. (presentation) Comparative pathogenicity of Dermo (*Perkinsus marinus*) between the Caribbean and American oyster. Int'l. Symp. Aquatic Animal Health, New Orleans, LA, 1-5 September 2002.

Scarpa, J., Laramore, S.E. and Bushek, D. (presentation) Comparative resistance between Caribbean *Crassostrea rhizophorae* and American *C. virginica* oysters to Dermo disease *Perkinsus marinus*. Aquaculture America 2002, San Diego, CA, 27-30 January 2002.

FUNDING PERIOD: _1998-2001__

PROJECT TITLE: Optimum Size for Planting Hatchery Produced Oyster Seed

PRINCIPAL INVESTIGATOR: Richard K. Wallace
AFFILIATION: Auburn University Marine Extension and Research Center
CO-INVESTIGATORS and AFFILIATIONS: David B. Rouse: Auburn University, Department of Fisheries and Allied Aquacultures

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≤ PROJECT RESULTS: Hatchery produced seed has not gained acceptance in the Gulf of Mexico Region primarily because survival of seed is poorly understood, particularly in relation to cost. Four size classes (5 mm, 6-10 mm, 11-15 mm and 16-20 mm) of two types of oyster seed (set on microcultch and set on whole shells) were planted at two sites (high and low salinity) in replicated plots during 1999. Three size classes (<15 mm, 15 - 25 mm and > 25 mm) of both types were planted in 2000. Oyster seed set on microcultch did not survive regardless of size, location or year, while survival of seed set on whole shell ranged from 42 to 62% after 32 weeks at the low salinity site in 1999 but did not survive in 2000. Drought conditions in 2000 resulted in the low salinity site being higher in salinity than expected and oyster drills were prevalent.

In laboratory predation experiments, oyster drills ate more large (> 25mm) single oyster seed than the other two size classes (< 15 mm and 15-25 mm) and than seed set on whole shell. When oyster drills were provided oyster seed set on whole shell only, they ate more of the largest size class. A similar study using blue crabs resulted in more medium and small oysters being consumed than the largest size class and more single oysters were consumed than those set on whole shell.

Survivorship of the three size classes of single oyster seed when exposed to hypoxic conditions (dissolved oxygen 0.01- 0.23 ppm) in a series of laboratory tests ranged from 100% after 72 hrs to 0% after 144 hrs. The mean estimated LT-50's for the three size classes of oyster seed (<20mm, 22-34mm, >35mm) were 99.37 h, 93.62 h, 93.03 h respectively, with no apparent relation between size and survival.

Analysis of survival for oyster seed set on whole shell from year one of this study and the published price of oyster seed of various sizes indicated there was no advantage in paying more for larger seed. Even though the smallest seed had the lowest nominal survival, the cost per oyster after 32 weeks was still lowest for the smallest seed size (<5 mm). The cost per oyster of the largest seed (16-20 mm) after 32 weeks was about 5 times higher than the smallest seed. For the conditions encountered during this study and the range of seed oyster sizes used, there is no economic advantage in paying more for larger seed.

IMPACTS and/or BENEFITS: The Gulf of Mexico region is the largest producer of oysters in the US. Continued high production is dependent on many factors but some experts believe that more intensive oyster farming can play an important role in maintaining or increasing production. Hatchery produced seed is an important element of oyster culture and the information from this study can give guidance to oyster farmers who are considering buying seed oysters. Significant savings can be obtained by purchasing spat set on whole shell at a smaller, less expensive size and by carefully selecting sites for growout.

PROJECT PUBLICATIONS:

Wallace, R. K. , D.B. Rouse, F.S. Rikard, J.C. Howe, B.A. Page, D.B. Gruber and J.K. Dunne. 2001. Experiments in determining optimum size for planting hatchery produced oyster (*Crassostrea virginica*) seed. World Aquaculture Society Book of Abstracts. Aquaculture 2001. p.675.

FUNDING PERIOD: 9-1-98 to 12-31-99

PROJECT TITLE: Caernarvon & Oyster Farmers: What Happened and What It Means

PRINCIPAL INVESTIGATOR: Sharonne O'Shea
AFFILIATION: LSU Sea Grant Legal Program
CO-INVESTIGATORS and AFFILIATIONS: Erinn Neyrey, Joe F. Stevenson, James Wilkins, LSU Sea Grant Legal Program

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PROJECT RESULTS:
Legal disputes between the federal and state governments, who constructed and operated the Caernarvon freshwater diversion project, and oyster farmers whose, leased oyster beds were adversely affected by the project, were the topics of this research. The project's results were an unbiased, even handed legal analysis of the strengths and weaknesses of the parties positions. The analysis revealed that attempts by the state to retroactively alter rights under leases that existed prior to 1995 would probably be deemed unconstitutional by Louisiana courts. The researchers also determined that the legislature has the authority to require that the state be indemnified from damage claims by oyster farmers resulting from coastal restoration projects in all future leases. The project further analyzed the Oyster Lease Relocation Program that the state established to head off damage claims from the Davis Pond diversion project.

IMPACTS and/or BENEFITS:
The two sides in these disputes, the state and federal governments on one side and oyster farmers on the other, have become very polarized and entrenched in their positions. The purpose of the study is to provide a non adversarial analysis that would be impossible to obtain from advocates of either side. We are hopeful that if the parties are more aware of their "real" positions they will be more likely to reach a fair compromise. There is some evidence of that from the fact that there were no lawsuits in the primary impact area of Davis Pond and from the fact that the state is now considering other means to address oyster lease problems. Our constituents trust the Sea Grant Legal Program to provide to be an unbiased source of legal information.

PROJECT PUBLICATIONS:

Joe F. Stevenson, Louisiana's Oyster Lease Relocation Program: A Step toward Common Ground, Southern University Law Review, Vol. 28 No. 1, 19-41, 2000

The Avenal Lawsuits, Louisiana Coastal Law, Vol. 77, October 2000

User's Guide to Louisiana's Oyster Lease Relocation Program (Brochure published by Louisiana Sea Grant and distributed to oyster farmers)

FUNDING PERIOD: 9/1/99 to 8/31/2000

PROJECT TITLE: Legal Authority to Clean Up Oyster Beds Closed Due to Pollution

PRINCIPAL INVESTIGATOR: Erinn Neyrey
AFFILIATION: LSU Sea Grant Legal Program
CO-INVESTIGATORS and AFFILIATIONS: James Wilkins, LSU Sea Grant Legal Program

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PROJECT RESULTS: A thorough analysis of existing statutory, regulatory, and case law was conducted to determine whether authority existed under the Clean Water Act and Louisiana water quality laws to allow oyster farmers to require cleanup of oyster beds contaminated by violating those laws. The analysis determined that there were two methods oyster farmers could pursue under the CWA: Supplemental Environmental Programs (SEP) and the citizen suit provisions. Strategies were discussed that would be more likely to result in cleanup of polluted oyster beds. The CWA also provides a possible mechanism for restoring oyster beds through the Clean Water State Revolving Fund. This fund has been underutilized in Louisiana for projects that benefit shellfish. Under the Louisiana Environmental Quality Act the researchers concluded that the law can be interpreted to allow the Louisiana Dept. of Environmental Quality to require oyster bed cleanup though it had not previously been used for that purpose. In addition the recently adopted Beneficial Environmental Projects regulations give DEQ and the public a new avenue to seek remediation of environmental damage.

IMPACTS and/or BENEFITS:
The great majority of oyster farmers in Louisiana and the Gulf are unaware of the potential under the CWA and state water quality laws for cleanup and remediation of polluted oyster beds. The research funded by this project is intended to inform oyster farmers in this area of law so that they may take advantage of the opportunities presented. We have received feedback that there are several oyster farmers who may attempt to gain redress under the mechanisms described by this project.

PROJECT PUBLICATIONS:

Erinn W. Neyrey and M. Michelle Marney, Cleanup of Contaminated Oyster Beds Under the Clean Water Act and Louisiana's Environmental and Water Quality Laws. Journal of Natural Resources & Environmental Law, Vol. 16 No. 2, 179-202, 2002

List of Attendees

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