

**ABBREVIATED PROGRESS REPORT**  
**SIGNATURE PAGE**

**PROJECT CODE: 05-3**

**SUBCONTRACT/ACCOUNT NO.: 557223**

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

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

**PARTICIPANTS:**

**Funded**

Roxanna Smolowitz, DVM, Marine Biological Laboratory

Dale Leavitt, Ph.D. Roger Williams University

Sandra Shumway, Ph.D., University of Connecticut

Gary Wikfors, Ph.D., Northeast Fisheries Center

**Non-funded Participants**

William Walton, Ph.D., Cape Cod Cooperative Extension

Richard Kraus, Aquaculture Research Corporation

Leslie Sturmer, Multi-county Extension Agent III; Sea Grant Extension Program

## **PROJECT OBJECTIVES:**

1. Determine the occurrence and severity of QPX disease in the progeny of two strains of geographically distinct brood stock of hard clams originating from MA and FL at 7 different temperatures, sequentially over the two years in the laboratory, after:
  - a. No stress.
  - b. Stress caused by 4 months of low food availability and burrowing deprivation.
  - c. Acute, heat-induced stress.
2. Quantify hemocyte types/morphologies and functional ability, including phagocytic ability, in the hemolymph of 2 strains of hard clams at:
  - a. Three seasons/temperatures (spring, summer and fall) in both QPX-infected and uninfected clams cultured in the field.
  - b. Seven temperatures (with both QPX-infected and uninfected hard clams) in the laboratory temperature stress experiment.

## **ANTICIPATED BENEFITS:**

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

## **PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:**

### **OBJECTIVE 1:**

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

**Clam Acquisition and Maintenance:** Seed from Florida (2 hatcheries) has been obtained. Because of the hurricanes in Florida in 2004, seed of the size original requested in the grant was in short supply to culturists and researchers alike. However, with the help of Leslie Sturmer (U. of Florida multi-county Aquaculture Extension Agent), Tom McCrudden (Research Aquaculture, Stuart, FL) and Joe Weissman (Clams R Us, Vero Beach, FL), approximately 28,000 hard clam seed (from Florida parent stock) of approximately 15 mm in shell height was purchased and shipped to the MBL. It was held at the Marine Resources Center (MRC) at the Marine Biological Laboratory until health certifications were finished, and until planting could be arranged. During this time, sea water that came into contact with the clams was treated to prevent spread of any disease. During the first 7 days, the static water used to cover the clams was chlorinated, heated to 60 EC, filtered to 2  $\Phi$ m, then

discharged into the fresh water disposal. For the following 3 weeks, the ambient flowing water was diverted to a sump treated with chlorine and ozone. Hard Clam seed from Aquacultural Research Corporation (ARC) was also obtained and a health certification was conducted. One hundred animals from each group of seed were examined as part of the health certification. Since leases in Wellfleet recently were identified with QPX, and because of the problems associated with that new finding, it was no longer available as a negative QPX site. Therefore, with the guidance of Tom Marcotti (Town of Barnstable shellfish warden), we selected an intertidal site in Barnstable Harbor that to this date has not shown hard clams positive for QPX but in which clams have previously been cultured (Rendevous Bay). Control animals were planted in that site. The potentially positive QPX plots were located in the Town of Barnstable intertidal culture area adjacent to Scudders Lane. Both locations provide easy access to the plots at low tide. Three 7 ft. x 7 ft. plots were planted with clams from each clam origin at each location. An additional 3 plots were also planted with extra clams at each location. Density on all plots was 50 animals/sq. ft. Plots have been covered with conventional netting held down with rebar rods and staples since planting. The plots are being maintained (with one net change this spring) by a Residential Americore volunteer working with Dr. Walton and watched over by the Town of Barnstable shellfish department. Temperature is being continuously monitored with Onset Continuous Temperature Monitors at each site. Additionally, Dr. Walton has deployed a YSI probe in Barnstable Harbor that has a direct web site link (it is deployed only during the summer). This link is <http://www.ysieconet.com/public/WebUI/Default.aspx?hidCustomerID=88>).

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

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

#### **OBJECTIVE 1.a. No Stress**

The first set of laboratory experiments began October 3, 2005. Six 12" x 12" plastic containers holding 1" of sand and approximately 2 liters of filtered sea water, were fitted with aerators and placed in a large tub containing recirculating, or static (depending on temperature required), fresh water that was either warmed or cooled to one of the temperatures noted above. 50 clams of either Florida or Massachusetts origin were placed in each of the 6 containers (3 of containers of each type) in each tub. Animals were acclimated for 2 weeks before the experiment began. After two weeks, 100 of each type of clam (FL

and MA) were injected with 0.5 ml of QPX culture in a dilute media and 50 of each type of clam were injected with media diluted 1/10 with sterile sea water before placement in the containers. Samples were removed at 1, 2 and 3 months. Examination of injected clams from the first sample period showed some live QPX in tissues of several animals, but no progressive infections in any animals. The final two sets are still being evaluated. The experiment did not run for the 5 months as stated in the grant because the animals were experiencing low to moderate mortality in the tanks, and the additional months of evaluation would not provide appropriate data.

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

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

**OBJECTIVE 2:**

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

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

The second sample period for field planted animals was conducted in May, 2006. The water temperature goal was missed by 6 days. The temperature at collection was to be 13E C. However a severe and quick warming event shot the temperatures up to 18E C approximately 6 days before the sampling was scheduled. The animals therefore experienced 6 days of increasing temperatures (from 13 to 19E C before sampling occurred. All animals were sampled from the plots in the harbor the evening before processing. After retrieval, animals were quickly transported to temperature controlled containers where they were held till processing on the following day. The processing was set up in the Barnstable County Farmhouse. The county and Bill Walton donated the use of the building and the labor of 2 people for the day. In addition to Dr. Wikfors, and Helene Hergaret who conducted the analyses and Dr. Smolowitz and her technician, a total of 1 undergraduate student from U. CONN, 3 student aquatic interns spending the semester at the MBL (from Roger Williams

University and Cape Cod Community College) and 1 Residential Americore representative spent the day bleeding 400, 2.5 cm shell height, hard clams to obtain hemolymph for FACS evaluation. In addition to collection of hemolymph from each animal, measurements and weight were taken as well as tissues for histopathological analysis. Wikfors and Hegaret brought the mobile FACS to the county farm house and ran each of the samples as they were collected. The next sampling period for the field work is scheduled for August 15/16, 2006. The temperature is expected to be at approximately 24E C at that time.

### **Immunological Analysis:**

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

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

- a.) Hemocyte viability, as a percentage of dead hemocytes
- b.) Phagocytosis of fluorescent beads by hemocytes, which stimulates the engulfment of non-self particles
- c.) Respiratory-burst response in hemocytes, that measures reactive oxygen species' potential to kill non-self particles previously engulfed by hemocyte and its ability to be induced by extra cellular products (ECP) of bacteria or inhibited with DPI.
- d.) Adhesion capacity of the hemocytes.
- e.) Percentage of apoptotic hemocytes.

### **Results to date:**

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

**WORK PLANNED:** Laboratory work as described in Objective 1.c. will begin soon after 1.b. is completed. Field sampling and hemolymph evaluation of animals planted in Barnstable plots will occur again in the fall when the water temperature reaches approximately 13EC. Additionally, Dr. Smolowitz's laboratory is collecting samples of the water column above the plots of clams in both the infected and control locations in Barnstable Harbor to evaluate using a real time QPX testing method. That data will be integrated with the pathological evaluation of the animals to help answer the question: Is occurrence of QPX organisms in the water column predictive of the QPX infection status of the clams in the plot. The next sampling period for the field work portion of the grant is

scheduled for August 15/16, 2006. The temperature is expected to be at approximately 25E C at that time (the highest temperature attained by the harbor waters). Water column samples for real time PCR evaluation are being taken monthly during the spring, summer and fall.

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

**PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:**

1. Barnstable County QPX Aquaculture Meetings. One was held in Wellfleet, MA and one at the Courthouse in the Town of Barnstable (during May, 2005). At these meetings (the idea for which originated from Smolowitz), the researchers met with town shellfish wardens, county representative and culturists to explain ongoing (and planned) work for areas affected by QPX. The work to be conducted in this grant was explained during those meetings.
2. Objectives of this grant were presented as part of a talk by Smolowitz at the NSA conference in Monterey, CA in 2006.



Legend: Dr. Wikfors demonstrating the use of the FACScan to clam bleeders at the Barnstable Farm house.