2.1 Objectives

1. Identify virulence factors expressed in QPX and associated mucus (extracellular proteins)
2. Characterize functional (immunological, morphological) and genetic factors responsible for hard clam disease resistance
3. Communicate with regional growers and managers to facilitate success of the hard clam industry

2.2 Justification

2.2.1 Economic Importance

The culture of marine shellfish and finfish makes up a large percentage of seafood production and is the fastest growing segment within the aquaculture industry (FAO 2001). Unfortunately, the U.S. is relying on seafood imports to meet their seafood demand. According to the FAO, U.S. consumers spent an estimated $52.3 billion for fisheries products, importing $9.0 billion of edible seafood in 1999. In contrast, the U.S. only exported $2.8 billion in fisheries products for a deficit of $6.2 billion. The U.S. trade deficit in seafood is the largest for any agricultural commodity and the second largest, after petroleum, for any natural resource product (Tlusty, Bengston et al. 2001). This, in part, has caused the U.S. Department of Commerce to call for a 5-fold increase in aquaculture production by 2025. The northern quahog (hard clam, *Mercenaria mercenaria*) is an economically-important U.S. seafood product, both as a wild caught and cultured bivalve species and could contribute significantly to the U.S. seafood production in the U.S.

In 1995, Quahog Parasite Unknown (QPX) was identified as the cause of major mortality in aquacultured hard clams in Provincetown and Duxbury, MA (Smolowitz et al. 1998). As of March, 2005, QPX has been identified as a significant cause of hard clam mortality in Virginia (Ragone-Calvo 1998), New Jersey (Ford et al. 2002), New York (Dove et al. 2004) and Rhode Island (personal communication, M. Gomez-Chiarri, U. Rhode Island). QPX has caused significant disease and mortality in either, or both, cultured and wild clam populations in all of these states. Movement of seed and adult clams between states and within localities of the same state has been heavily restricted (personal communication, M. Hickey, Dept. Marine Fisheries, MA and L. Sturmer, Multicounty extension agent, Florida). Transplantation of wild, native clams, for depuration and eventual commercial harvest, has been prohibited because of positive disease findings in New York. It has been estimated that closure of Raritan Bay to the NY Shellfish Transplant Program has resulted in a loss of 45% of New York's annual hard clam production (D. Barnes, NY State Dept. of Environ. Conservation). This has resulted in a loss of several million dollars to the NY hard clam industry. Recently, two locations in Massachusetts (Barnstable and Wellfleet) were severely impinged upon by this disease and substantial mortalities occurred in both areas. Wellfleet, a prime aquaculture area for clams experienced its first identifiable outbreak of QPX in the fall of 2004. In an attempt to prevent the disease from spreading to other areas within the aquaculture site, aquaculturist in the affected area (Egg Island, a sand bar in Wellfleet Bay) removed all the clams planted in that area. This recent disease outbreak represents a loss to the Cape Cod clam culturists of over a million clams resulting in losses well over $200,000 dollars in product and at least three times that in future production. This loss results from direct losses as well as unrecoverable time invested and infection of aquaculture leases (personal communication, William Walton, Cape Cod Cooperative Extension).

2.2.2 QPX

QPX is a protist, genetically identified as belonging to the family Thraustochydriidae in the phylum Labyrinthulomycota (slime mold). It proliferates by endosporulation (Kleinschuster et al, 1998; Mass, et al.,1999; Smolowitz et al., 1998). In Massachusetts, although much less often in other states, chronic, proliferative, QPX-containing, inflammatory nodules and irregular swellings are noted in the

mantles of up to 75% of the infected clams lying on top of the sediment (unpublished data, Smolowitz). Microscopic examination of squashed tissues from these foci have revealed the presence of QPX organisms and accompanying intense inflammation (Smolowitz, et al., 1998).

Immune responses of clam hemocytes to QPX and its mucoid secretions have been studied under controlled, in vitro laboratory conditions. It is likely that the mucus may reduce contact with QPX, preventing subsequent recognition and uptake. However, it is possible that certain extracellular proteins (ECPs) within the mucus are involved in down-regulating phagocytosis. Work in Anderson's laboratory suggests that the pathogenicity of QPX resides both with mucus production and with the ECPs secreted in the mucus of the parasite. For example, we have shown that QPX ECPs function as inflammatory agents causing hemocyte invasion of foci of infection, protective agents against serum-borne anti-QPX molecules such as the clams proteolytic enzymes, and hemocytic phagocytosis. Therefore, it is necessary to fully characterize QPX ECPs as virulence factors involved in potential immunological suppression. To this end, we plan to identify QPX ECPs and study their expression on a genetic basis as part of our first research objective. Additionally, the effects of ECPs secreted by QPX on particular key immune parameters will be characterized as part of the second research objective of the current proposal.

2.2.3 Evidence and mechanisms of QPX resistance in hard clams

In the mid-1990s, MA clam growers and scientists anecdotally noted significant differences in QPX disease survival among clam strains originating from geographically distinct areas that were later grown at the same site. New Jersey clam stock suffered much higher mortality than local seed when grown on Cape Cod, MA. In New Jersey, South Carolina clams suffered higher QPX mortality than New Jersey clams (Ford et al., 2002). During a 3-year study, clam strains produced at VIMS from brood stocks originating from Massachusetts, New Jersey, Virginia, South Carolina, and Florida were grown at sites in Massachusetts, New Jersey and Virginia and evaluated for survival, growth, condition, and QPX disease susceptibility. The clams originating from South Carolina and Florida brood stocks had significantly higher prevalence of QPX and higher mortalities than clams originating from Virginia, New Jersey and Massachusetts brood stocks when grown in Virginia (Ragone-Calvo et. al., 2003). Importantly, these findings were mirrored in the New Jersey location. Unfortunately, "overwintering mortality" (not QPX-caused) occurred in the Massachusetts location disrupting this experimental site. This study established that seed clams spawned from a more southern brood stock (in relation to where the clams were planted) developed higher prevalence of QPX disease and suffered higher mortality than seed from brood stock of local or more northern origin. These data strongly suggest that resistance may be tied to winter hardiness of the strain.

Histologically, the clams usually mount an intense, encapsulating, hemocytic, inflammatory response (granuloma formation in mammalian terms) to the QPX organisms. Encapsulation reflects an invertebrate's attempt to form a hemocytic wall around organisms that are either too large to be phagocytosed by individual hemocytes or that produce an inhibiting substance (i.e. mucus) (Anderson et al., 2003, Cheng, 1996). In QPX infections, giant cell formation is usually seen only in areas of intense inflammation that contain moderate to few remaining QPX organisms and reduced amounts of QPX mucus. This unusual cell is thought to represent the joining of several hemocytes to form a mega-cell capable of phagocytosis of organisms intermediate in size between those a single hemocyte could phagocytize and those infectious organisms that could only be contained by the encapsulation process involving layering of large numbers of hemocytes. The importance of giant cells, conditions of its formation and potential genetic propensity for its formation, to the occurrence of QPX infection is not known and should be investigated.

The typical hemocyte response to a microorganism first involves immune recognition followed by ingestion (phagocytosis). Identifying temperature-related variation in the function of clam

hemocytes would elucidate factors involved in the inflammatory response (or lack of) that result in disease. It has been reported that hard clams are physiologically most active between 20 and 25°C and are dormant during the winter (Grizzle et al., 2001). Combined effects of environmental variation and physiological adaptation status could result in a vulnerable period that would allow the organisms to establish an infection in the tissues because of potentially retarded hemocyte function, especially phagocytic abilities, in the spring and fall especially if some strains are less adapted to the cold than others. We have preliminary (unpublished) microscopical evidence that viable QPX washed free of its mucoid coat can be phagocytized by clam hemocytes. Washed QPX (wQPX) are probably destroyed after ingestion, again based on direct observation. The QPX organism is characteristically enveloped by mucus so it is this condition upon which most immunological effort will be spent.

Hemoctytes are considered to be the immune effector cells of bivalve mollusks; they either phagocytize and kill pathogens intracellularly, or they secrete peptides and other molecules that exert their antimicrobial activity outside the cells in the serum. Hemocytes of some bivalves may kill pathogens using reactive oxygen or nitrogen species (ROS, RNS) and/or lysosomal hydrolases. In many bivalves, phagocytosis triggers assembly/activation of the membrane-associated respiratory burst enzyme, NADPH oxidase, which catalyzes the generation of superoxide anions (Q^{-}_{2} from the reaction of NADPH and molecular oxygen (Anderson 1996). Superoxide is rapidly converted to hydrogen peroxide (H_2O_2) spontaneously, or via superoxide dismutase (SOD), which may then be converted to other highly toxic ROS used by phagocytes to kill ingested microbes (Horan et al. 1982). The hemocytes of oysters and mussels contain the MPO-H_2O_2-Cl antimicrobial pathway (Klebanoff 1968), as shown by the measurement of luminal-augmented chemiluminescence (Larson et al. 1989, Anderson et al. 1994). It is interesting that this pathway is not as active, or lacking, in clam species (Anderson 1994; Greger et al. 1995). ROS apparently are not produced by *M. mercenaria* hemocytes during phagocytosis of various test particles, including QPX (Anderson et al. unpublished.); however, the role of RNS (such as nitric oxide) in anti-QPX defenses has not been defined. Nitric acid (NO) is an antimicrobial radical produced by the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS). The interaction of NO with O^{-}_{2} will form peroxynitrite (-OONO), a powerful cytotoxic agent comparable in reactivity to the hydroxyl radical. It is thought that the RNS system is phylogenetically conserved because NO is found in the hemocytes of the horseshoe crab, Limulus polyphemus (Radomski et al. 1991), as well as snail and mussel hemocytes (Ottaviani et al. 1993). In mammalian species, NO is known to be particularly effective in destroying intracellular parasites and extracellular pathogens. Therefore, NO could play a role as a defense molecule involved in responses to QPX and other bivalve pathogens.

A classical antimicrobial agent found in bivalves is lysozyme (McDade and Tripp 1967), a lysosomal enzyme that preferentially lyses the cell walls of gram-positive bacteria, may have opsonic properties and may be active against bivalve parasites such as *P. marinus* (Chu and La Peyre 1989). Lysozyme in molluscan sera is probably a result of degranulation of hemocytes during phagocytosis, and its titer can be increased by various hemocytic stimuli (Anderson 1984); this has been shown to be the case for *M. mercenaria* (Cheng et al. 1975).

Phenoloxidase (PO) has long been associated with microbial destruction in anthropods such as crustaceans (Söderhall et al. 1994). PO is the terminal enzyme in the prophenoploxidase (PPO) system, a complement-like enzymatic cascade responsible for melanin synthesis. Melanin deposition may be associated with encapsulation of invertebrate pathogens, and quinines produced as by-products of melanin synthesis have antimicrobial activity. PPO activation also produces factors involved in non-self recognition (opsonins) and cell adhesion proteins. Trypsin-inducible PO activity was initially not detected in molluscan species (Smith and Söderhall 1991), apparently due to a technical detail (the use of certain anticoagulants), but a number of reports of PO activity in mollusks have subsequently

appeared. *Mytilus edulis* hemocytes contain peroxidase and PO; PO activity in this species can be stimulated in vitro by zymosan (Coles and Pipe 1994). Similar studies by Renwrants et al. (1996) showed PO by the conversion of L-dopa to melanin in *M. edulis* hemocytes, but this activity was not seen in all cells examined. Subsequent papers report PO in a marine mussel, *Perna viridis* (Asokan et al. 1998), in *Mytilus galloprovincialis* (Carballal et al. 1997) and in *Geukensia demissa* and *M. mercenaria* (Deaton and Dankert 1998).

Clearly, QPX is a major concern for the hard clam industry in the Northeast United States. In order to develop prevention and management strategies for this emerging disease, it is necessary to gain a thorough understanding of the host-parasite interaction at the molecular and immunological level. The first objective is designed to understand mechanisms of QPX virulence by identifying expressed genes. Previous work by Anderson has demonstrated extracellular proteins in the mucus are virulence factors. Once specific genes associated with virulence of QPX have been identified, the presence and relative virulence of QPX in the environment could be easily evaluated. Investigators on the current proposal and other collegues (R. Gast; WHOI) have already developed advanced molecular assays for detection of QPX via rDNA. With the ability to simultaneously quantify QPX gene expression (RNA) in conjuction with rDNA, we will not only be able to detect the presence of QPX but have the ability to measure the proportion of live vs dead organisms, which strain(s) are present, and the relative virulence of the local population. In addition to facilitating the development of monitoring technologies, the information provided regarding how temperature and host tissue influences virulence will give growers information useful in resource management (e.g. site planning, density).

The other major focus of the proposed research is the functional (immunological, morphological) and genetic factors responsible for hard clam disease resistance. Similar to the research proposed to examine QPX virulence, analysis of hard clam hemocyte immune responses will primarily be conducted under controlled laboratory conditions. Therefore, upon completion of the proposed research we will certainly produce direct data on hard clam QPX resistance. Well designed field trials are beneficial to continued development of the industry, however, independent field based experiments are vulnerable to unpredicted natural phenomenoms. In addition, it is often complicated to extrapolate results associated with specific factors (i.e. QPX) if complementary lab-based experiments are not carried out. Once genes associated with QPX survival, giant cell formation, and phagocytosis have been characterized in hard clam hemocytes, we will have the ability to screen different strains of clams in order to assess their potential QPX resistance. This will immediately assist growers in deciding which strain to use. Additionally we will be able to better understand the fundamental reasons why select strains used in field trials (e.g. Kraeuter's NRAC proposal) have higher survival rates. In the long-term, a complete understanding of bivalve disease resistance will allow for the expedient development of superior broodstock / lines in all commercially important species.

2.3 Related Activities and Other Work

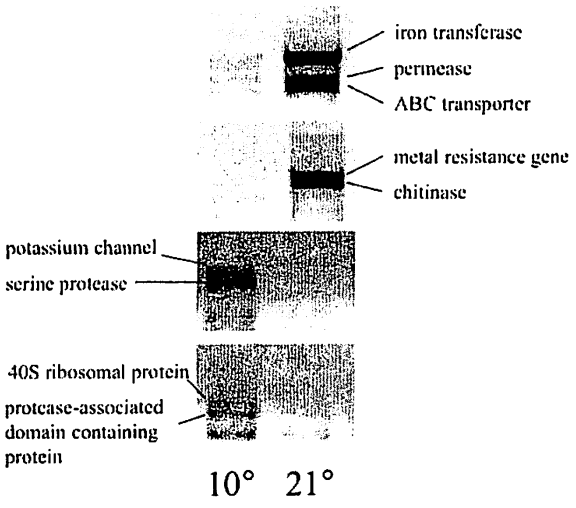


Figure 1. Gel electrophoresis of differentially expressed genes expressed by QPX at two temperatures. Putative identities are based on sequence comparisons with BLASTX database (NCBI)

In order to begin to understand what genes are present in the extracellular proteins of QPX mucus and are associated with QPX virulence, we have started to characterize genes that are differentially expressed by QPX in relation to temperature. For this initial experiment, QPX cultures were grown at 10° and 21° for 10 days. Following incubations, total RNA was extracted from cultures and several differentially expressed genes were identified. The molecular techniques involved in this experiment are identical to what is proposed in the current grant and are described in detail below (Proposed Methods and Activities). Briefly, genes (protein coding portions of genomes) are arbitrarily amplified using proprietary primers in samples to be compared. The advantage of this system is that there is no limit to the number of samples that can be compared, making it very suitable for the proposed study where several factors (e.g strain, temperature, time) are compared . We found several differentially expressed transcripts in QPX incubated at different temperatures (FIGURE 1). PCR products were excised from the agarose gels and sequenced. Putative identification of these genes is indicated adjacent to the PCR products based on top Blast (NCBI) hits. In other words, identities are based on sequence comparison with other known genes. To our knowledge, these are all novel gene homologs in QPX as only rDNA sequences have been characterized in the past. Even though these data are preliminary, several of these genes provide insight into pathogenecity of QPX. Identification of a putative iron transferase suggests iron could be regulatory signal for the expression of virulence factors. This regulatory pathway has been observed in several host-parasite relationships (Aguila and Brock 2001). In addition to the utilization of iron for regulating virulence factors, it has also been shown simply interfering with iron metabolism in the host organism can negatively effect host physiology (Aguila and Brock 2001). Pore-forming proteins such as potassium channels identified as part of this preliminary experiment, are also important for pathogens as they have been shown to inhibit phagocytosis and escaping phagsomes (Almeida, Noronha et al. 2002). For the proposed study we will continue using this approach to fully understand what factors (proteins) are expressed and how they might effect hard clams.

Ongoing work on QPX are investigating the effects of temperature on infection of hard clams by QPX (Smolowitz, Leavitt, Shumway, Walton and Wifors, funded by NRAC) in a laboratory setting. Information concerning the effects of temperature on development of disease can be directly used in the work proposed in the present submission. Another grant titled Development and application of molecular methods for detection of QPX organisms in the environmental reservoirs (Gast, Smolowitz, Leavitt, Reece, 2004-2006, WHOI Sea Grant) is using PCR to identify QPX in the environment.

A grant titled: Evaluation and Genetic Analysis of hard clam, *Mercenaria mercenaria*, stocks for QPX-resistance by Kraeuter et al., is being submitted to NRAC parallel to this grant. We will confer directly with P.I. and other researchers on this grant (especially Ximing Guo) to compare data and identify important findings concerning temperature effects and gene discovery based on hemocyte activity. If Kraeuter's NRAC proposal is funded, clams used in those field trials will be a primary source of clams used for validation and application of genetic markers developed as part of objective 2 of the current proposal.

2.4 Proposed Methods and Activities

2.4.1 Objective 1: Identify mechanisms of QPX virulence

In order to elucidate specific proteins produced by QPX that are pathogenic to hard clams, a set of experiments will carried out where pure cultures of QPX are incubated in the presence and absence of clam tissue at three temperatures (10°, 21°, and 24°). These combined conditions will allow us to examine differences in both mucus production and changes in QPX gene expression. A minimum of 4 strains of QPX will be used for this objective. The Smolowitz laboratory currently has 6 different confirmed/or potential QPX isolates in culture (originating from Barnstable, Pleasant Bay and Wellfleet, MA) and one isolated in continuous culture for approximately 6 years originating from Provincetown, MA). Treatments that include clam tissue will include a small (0.5 mm) clipping of mantle tissue. Each treatment will be performed in triplicate and mucus quantitation and RNA samples will be taken at 24 hours and 10 days. By studying the comparative physiology of QPX under these various conditions, we will provide information what components of QPX and associated mucus are virulent.

Specific Protocols

QPX Culture

Intact nodules from infected clams are excised, washed three times then incubated at 4°C overnight in autoclaved, 0.2 μm-filtered natural seawater containing 100 units penicillin and 0.1 grams streptomycin per ml. The next day, using sterile technique, nodules are washed three more times in the aforementioned solution. Each nodule is place on a sterile microscope slide, the fluid contained within the nodule expressed, and 100 μl of this QPX-containing fluid was transferred to a 25 cm2 cell culture flask with 10 ml of culture medium (Kleinschuster, S.J., R. Smolowitz, and J. Parent, 1998: MEM Eagle (α modification, Sigma 0644) 5.1 g/L; CaCl2 x 2H2O, 1.82 g/L; KCl, 0.68 g/L; MgCl2 x 6H2O, 4.36 g/L; NaCl, 24.26 g/L; MgSO4 x 7H2O, 3.16 g/L; HEPES, 5 g/L; glucose, 0.5 g/L; heat-inactivated fetal bovine serum, 10% by volume; pencillin, 100 U/ml; and streptomycin, 0.1 mg/ml) added. Cultures are maintained at ambient room temperature (25°C) under normal atmospheric conditions. Following initial inoculation, each culture is monitored daily to avoid bacterial or fungal contamination occurred. Each cultured isolate is sub-cultured on a weekly basis. Sub-culturing entails aseptically transferring 250 μl of the parent culture to a new 25cm2 cell culture flask containing 10 ml of fresh medium.

Differentially Expressed Gene Isolation

First QPX will be harvested from cell culture flasks and total RNA will be extracted (Chomczynski, 1993, Chomczynski, 1987). The GeneFishing DEG System (Seegene) will be used to isolate late genes that are differentially expressed under different conditions (i.e. QPX strain, clam presence, temperature and time). This system is based on Annealing Control Primers (ACP) technology. The reason this approach is being used is that it is economical, fast, and easily managed to compare a large number of different samples. The principle of ACP technology is based on the tripartite structure

of a specific oligonucleotide primer (ACP) having 3'- and 5'- end distinct portions separated by a regulator and the interaction of each portion during two-stage PCR. The resulting PCR products will be run on an agarose gel, and differentially expressed bands removed. PCR products will be cloned into TOPO TA pCR 2.1 (Invitrogen) and positive colonies grown for plasmid DNA. Templates will be prepared in a Rev Prep Orbit (GeneMachines) and the resulting cDNAs sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions will be precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems). All sequences will be analyzed by NCBI Blast programs for similarity to known genes (Altschul, 1997). ClustalW (MacVector 7.2) analysis will be used for sequence pair-wise and multiple protein alignments. Once gene products have been identified through DNA sequencing, quantititative RT-PCR will be used to confirm differential expression. Quantititative PCR will include characterizing genes of interest (e.g. iron transferase) in different strains of QPX that have been isolated by Smolowitz. This will validate expression differences and quantitate putative differences in strains

2.4.2 Objective 2. Characterization of functional and genetic factors responsible for hard clam disease resistance

Research associated with this objective involve characterizing the fundamental immune response of hard clam hemocytes in response to QPX exposure. Specific parameters that will be evaluated and were previously described (Section 2.2.3) include phagocytosis, RNS generation, anti-QPX activity (killing), lysosyme activity, and PO activity. In order to characterize these various functional responses, hemocytes harvested from select strains of clams will be cultured in the presence of QPX (culture techniques described above). Because of the logistics and costs associated with this analysis only one strain of QPX will be used. Hemocytes will be harvested from the same strains of clams proposed for use in Kraeuter's NRAC propoposal (South Carolina, New Jersey, and Massachusetts). In addition to assessing the functional immune response of hemocytes to QPX exposure, genes involved in exposure / resistance will be determined using the same protocol used for isolating QPX expressed genes (DEG-SeeGene). Upon completion of these combined experiments, we will be able to better understand defense mechanisms involved in QPX exposure and how these vary across strains and with temperature. Ultimately genes identified will be used in marker-assisted selection programs and help growers and scientists better understand why a particular strain of clam is superior in a given location.

Specific Protocols

Phagocytosis.

Fluorescein isothiocyanate (FITC-conjugated zymosan and trypan blue quenching dye are prepared according to the method of Hed (1986). QPX organisms will be FITC-labelled by the same method used in this lab tro label P. marinus and various bacteria used in other phagocytosis studies. FITC-labelled particulates are incubated (20°C, 30 min) with hemocytes in primary cultures, during which time phagocytosis proceeds. Ice-cold trypan blue is added to quench the fluorescence of uningested FITC-labelled QPX or zymosan, and the fluorescence of the ingested particles quantified with an FCA (fluorescence concentration analyzer, $\lambda ex/\lambda em = 485/535$. The assay is done in 96-well microtiter plates as described by Wan et al. (1993) and Poszell and Andersopn (1994). The ability of clam plasma QPX agglutinins to promote phagocytosis (serve as opsonins) will be carried out.

RNS Generation by Hemocytes.

NO (nitric oxide) will be determined in hemocytes primary culture supernatants (hemocytes will be exposed to QPX during primary culture) using the Griess reagent, positive responses produce absorbance at 540 nm. This assay will be performed in microplate format, using kit NB 98 (Oxford Biomedical Research) which permits measurement of as little as 1 pmol NO/uL . NOS (nitric oxide synthase) will be detected by boiling hemocytes in SDS buffer for 10 min, lysates are centifuged (16,000 g, 5 min) separated by SDS PAGE and the bands transferred (4 h, 100) to a PVDF membrane (Fluorotans W. Pall Corp.). Membranes are probed with a polyclonal antibody against a 21 kDa conserved fragment of mouse macrophage NOS (#N32030, Transduction Labs). Western blot analysis with this antibody shows a strong cross-reaction with a C. virginica protein of MW similar to mouse NOS (~130 kDa). Relative NOS concentrations will be approximated by densitometry using CL autoradiography and NIH Image.

Hemocyte Mediated Anti-QPX Activity

Killing will be measured colorimetrically by the enzymatic reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2-H-tetrazolium) in the presence of PMS (phenylmethosulfazone) as supplied by Promega Co. The assay will be performed in flat-bottomed 96-well microtiter plates. Hemocytes and QPX are incubates (25°C, 3 hr); surviving QPX are permitted a "grow-out" period in culture medium (the exact period needs to be determined in preliminary studies). MTS is reduced by the surviving QPX to a water-soluble formazan which can be read at 490nm in an ELISA reader. Hemocyte-only controls permit correction of the data for any contribution to total MTS reduction by the hemocytes. A killing index is then calculated from the difference in MTS reduction between QPX incubated with and without hemocytes. This method has been used to study microbial activity of bivalve hemocytes (Volety et al. 1999) and is conceptually similar to a MTT-based antimicrobial assay used in this lab (Roszell and Anderson 1996).

Lysosyme assay

Filter-sterilized plasma (0.1 ml) will be added to 3 ml Micrococcus luteus cell wall suspension (0.15 ug/ml) and the change in OD (450 nm) monitored for 30 min., The lysozyme titer will be expressed as units of egg white lysozyme activity, as calculated from a standard curve. The susceptibility of QPX to egg white lysozyme (at relevant serum lysozyme levels) will be determined. If significant QPX cidal activity is detected, M. mercenaria lysozyme will be isolated and used for subsequent studies.

Phenoloxidase and Peroxidase assay

Hemocytes are firmly attached to glass slides using the cytocentrifuge. One sample will be incubated in 0.5 mg/ml DAB (Sigma Chem. Co.) in phosphate-buffered saline (PBS) containing 0.02% H2O2 (35 min, 20°C). Another sample will be incubated (90 min, 30°C) in 1 mg/ml L-dopa in PBS. Control preparations will be incubated in the absence of DAB or L-dopa. Positive cells are indicated by a brown color (for peroxidase) or dark gray or black color (PO), as compared to control hemocytes. Percentage positive cells per sample will be determined after counting >200 hemocytes. Plasma samples will also be examined for PO, but not for peroxidase. The activity of 5thse enzymes, that are linked to host defense capabilities, will be determined in hemocytes (and plasma, if possible) before and after exposure to QPX. The analytical methods are based on those of Dyrynda et al. (1998).

Differentially Expressed Gene Isolation

In order to determine the genes involved in QPX resistance the same molecular techniques proposed to study QPX gene expression will be used (described in detail above). Specifically, hemocytes from clams (same strain of clams used in Kraeuter's proposal) will be incubated in the presence and absence of QPX. Following incubations of 24 hours and 36 hours, RNA will be extracted from the cultures and clam genes upregulated in the presence of QPX will be identified. These experiments will be carried out at three different temperatures (10°, 21°, and 24°). Sequencing and expression profiling will be carried out as described for QPX differentially expressed gene isolation.

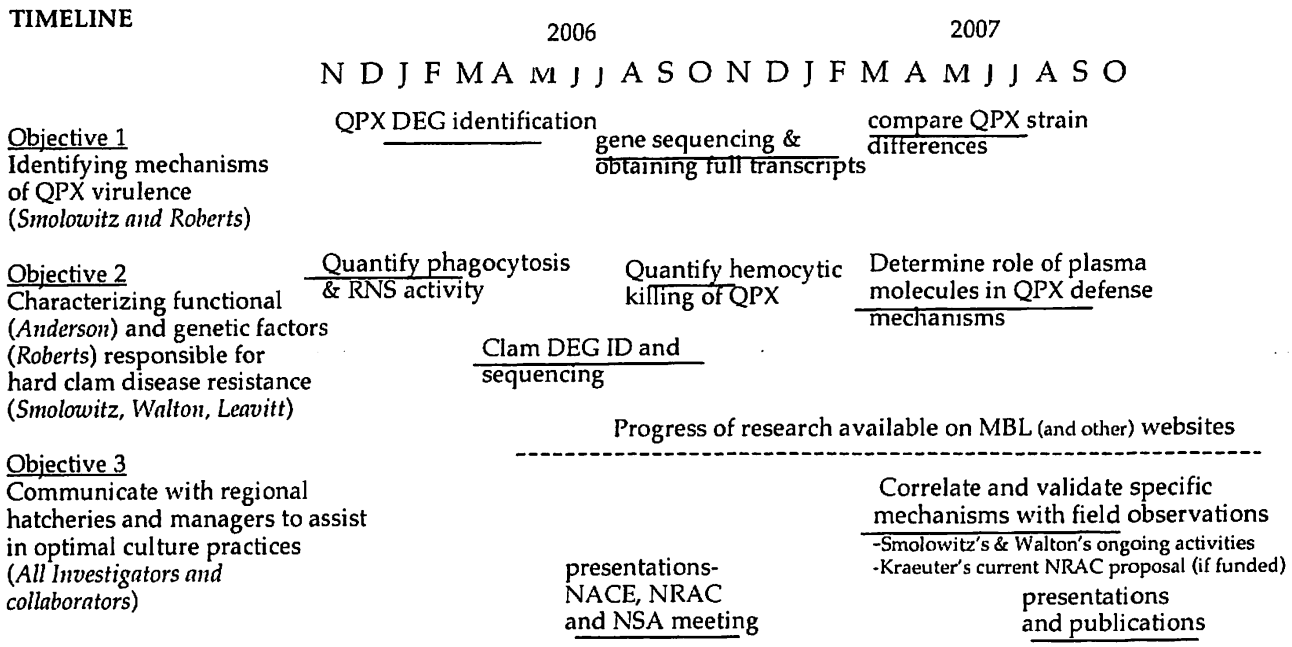
2.4.3 Objective 3. Communicate with regional growers and managers to assist in optimal culture practices

The last objective of the proposed study includes working with industry and research based growers to validate the traits associated with QPX resistance in clams. Upon completion of research objective 2, we will have a comprehensive understanding of the functional and associated genetic factors involved in resistance. During the second year of the proposed study we will sample clams strains used in other research projects (i.e. PI – Smolowitz, PI – Kraeuter) and used by regional hatcheries (obtained through Walton and Leavitt). At this time QPX exposure and putative resistance will be determined for each source as a result of research carried out under controlled laboratory conditions, well design field trials, and/or documentation of QPX outbreaks. In cases were differential survival is observed, genes isolated as part of objective 2 will be quantitated to validate the use of these genes as indicators of resistance. For example, if during Dr. Smolowitz's currently funded NRAC project, strain "X" experiences 100% mortality upon exposure to QPX and strain "Y" demonstrates significant resistance; hemocyte samples will be taken from both strains. Total RNA will be extracted and real-time quantitative RT-PCR will be carried out to validate relative importance of genes identified using DEG.

In addition to communicating our findings relating to clam resistance we will also be in touch with growers and managers to evaluate virulence of different strains of QPX. As part of their routine activities Smolowitz and Walton are immediately aware of QPX outbreaks in Massachusetts and surrounding states. When outbreaks occur samples will be taken and genes identified that have been determined to associated with virulence will be quantified using quantitative RT-PCR. Based on the current work of the PIs, we know that environmental samples of QPX can be taken from water and genetically analyzed by filter concentrating. Samples will be taken during any outbreak that occurs during the proposed project, however analysis will not occur until the end of year two.

2.5 Project Schedule

Duration of the proposed study is two years with an estimated start date of November 2005. Research associated with objective 1 will be primarily carried out by Smolowitz and Roberts. The majority of this work will be performed during year one when experimental QPX incubations are carried out and differentially expressed genes are identified. Research associated with objective 2 will primarily be carried out by Anderson at CBL. Smolowitz will assist in providing QPX and clams. The various functional assays conducted by Anderson will be performed throughout the two years with phagocytosis and RNS activity assays carried out in the beginning of year one. At the end of year 1 Anderson will send RNA samples of hemocyte cultures for Roberts to identify potential markers for QPX disease resistance (DEG). During year two we will actively work with growers and managers to assess putative QPX resistance of clams and virulence of QPX strains. Results of research will be presented at meeting and workshops as well as outline on an MBL hosted webpage.



2.6 Anticipated Products/Outcome

The results of the proposed study will provide important information in expanding our understanding of the mechanism of QPX infection in hard clams. Characterization of the processes behind prospective clam resistance through characterizing the mechanisms inovlh by the QPX protist will provide important tool for hard clam hatcheries and ultimately the farmers. The most important tool is a mechanism to identify those clams that may have enhanced capacity to resist the actions of the QPX protest. By developing an ability to predict QPX resistance, we can use this knowledge to refine hatchery broodstock selection to enhance the resistance of the population of seed clams generated by a hatchery. Therefore, it is important to deliver the results of this study to a target audience of commercial shellfish hatchery managers and to growers.

The target audience(s) will utilize this information to direct the selection of appropriate broodstock to enhance hard clam resistance to QPX either directly, in the case of the hatchery manager,

or indirectly, through pressure from shellfish growers to adopt our current knowledge of brookstock selection in hatcheries. For this reason, the primary outreach effort in this study will be to translate the results of the study into an applied strategy for hatchery operators. Initially, the results of the study will be distributed to shellfish hatchery managers and to shellfish growers through our conventional outreach tools. This is primarily through presentation of the study results in venues where industry members will be in attendance, including the Northeast Aquaculture Conference and Exposition (to be held in Connecticut in 2007), National Shellfisheries Association annual meeting, and/or the annual Milford (CT) Aquaculture Seminar. Results will be communicated ay these venues by Roberts, Anderson and/or Smolowitz in at least 3 oral presentations or posters. Results will also be presented at several meetings for aquaculturists and regulators such as the Rhode Island Aquaculture Conference in 2008, workshops on Cape Cod (presented by the Barnstable County Shellfish Advisory Committee and SEMAC, organized by Walton), a workshop by MD Sea Grant organized by Anderson ands at the Massachusetts Shellfish Officers Association (Walton and Smolowitz). Leavitt has organized and conducted a number of dedicated workshops, since discovering QPX in the northeast, targeted at shellfish growers to inform them of our current state of knowledge about QPX disease in hard clams. If one or more of these workshops should be scheduled, we will present the results of this study as a component of our report on our advancing knowledge of QPX. In addition, we will construct a short information piece derived from the results of the study and distribute it to all commercial and research shellfish hatcheries within the range of the QPX disease (Maine to Virginia). We will use the annual East Coast Shellfish hatchery list produced by Mr. George Flimlin at Rutgers (NJ) Cooperative Extension, anticipated to be approximately 25 hatcheries. Lastly, we will offer to provide limited initial screening of selected hard clam lines from commercial hatcheries to evaluate their capacity to resist the protistan infection, based on the knowledge derived from this study. Walton will co-author a technical bulletin with the other PIs [presenting new findings from this work. The bulletin will target shellfishermen, aquaculturists and local natural resource managers, The bulletin will be sent to hatchery managers growing hard clams on the east coast, and it will be published on the NRAC website.

2.7 Supporting Facilities

Anderson. Office for P.I., five newly renovated standard laboratory modules. Gateway 2000 P5-120 and GP6-333 computers plus HP LaserJet IID printer. Available equipment includes: chemical fume hood, laminar flow hood, research photomicroscopes (2) e3ith phase, phase interference, dark field, fluorescence, etc., refrigerated centrifuge, ultracentrifuge, micro-balance, balance, spectrophotometer, fume adsorber, tissue disrupters, refrigerators, ultralow freezer, CO2 incubators, water baths, pH meter, bacterial plate counter, liquid scintillation counter with single photon monitor, IDEXX fluorescence concentration analyzer with dedicated computer, ELISA plate reader, Top Count 96-well plate reading liquid scintillation counter, electrophoresis equipment, BioRad Mini-Protean II units with 2-D gel capacity, preparative SDS-PAGE, Rotofor preparative IEF, vacuum transfer apparatus, thermal cycler, lyophilizer, and Pharmacia AKTA prime protein separation system. Large wet lab in controlled environment aquatic animal facility, recirculated and flow-through water systems, temperature and photoperiod controlled. Graphic arts, computer services, autoclaves, cold rooms, and other support facilities are available in house.

Roberts and Smolowitz. Equipment available at the MBL includes office and laboratory space in the Marine Resources Center (MRC) (a state of the art facility able to provide a continuous supply of raw or treated sea water). Smolowitz's laboratory contains exhaust hoods, a biological hood, an Olympus dissecting microscope with camera attachment, a digital 3030 Olympus zoom camera, ovens, refrigerators, EPIC PC systems computers with printers, a multiheaded (3) Zeiss Axioskop 2 with attached Olympus DP11 digital cameral and a Zeiss MC80 conventional camera. All molecular work (S.Roberts) will be performed in a 800 sq.ft. research laboratory in the MRC. The lab is equipped with routine laboratory furniture, plumbing, chemical and biohazard hoods, and computers. It is also completely equipped to do all the recombinant DNA work necessary to develop the Real-time assay and to process and analyze samples with this assay. This includes in particular, a MJ Research Opticon Real-time system. In addition, MBL general use equipment includes ultracentrifuges, -80 freezers, spectrometers, etc. In MA, fixed, trimmed tissues will be processed into paraffin sections by an outside service laboratory (Mass Histology Service, Warwick, Rhode Island; www.masshistology.com). There is a core microscopy facility at MBL (http://www.mbl.edu/inside/what/services/serv_micro.html) containing various microscopes and histological processing equipment. There is a genome facility in the "Bay Paul" Center at MBL that has an ABI 3700 sequencer, automated array and reader and ancillary robotics.

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