Effect of Temperature on the Infection of Hard Clams (*Mercenaria mercenaria*) by the Protistan Organism Quahog Parasite Unknown

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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.

METHODS AND PROCEDURES:

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) was obtained. Because of the hurricanes in Florida in 2004, seed of the size original requested in the grant was in short supply to culturists and researchers alike. However, with the help of Leslie Sturmer (U. of Florida multi-county Aquaculture Extension Agent), Tom McCrudden (Research Aquaculture, Stuart, FL) and Joe Weissman (Clams R Us, Vero Beach, FL)., approximately 28,000 hard clam seed (from Florida parent stock) of approximately 15 mm in shell height was purchased and shipped to the MBL. It was held at the Marine Resources Center (MRC) at the Marine Biological Laboratory until health certifications were finished, and until planting could be arranged. During this time, sea water that came into contact with the clams was treated to prevent spread of any disease. During the first 7 days, the static water used to cover the clams was chlorinated, heated to 60 °C, filtered to 2 μm, and 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 evaluation was conducted. One hundred animals from each strain 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 at the date of planting had 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 provided easy access to the plots at low tide. Three 2.13 x 2.13 m (7 ft. x 7 ft.) plots were planted with clams from each clam origin at each location. Density on all plots was 50 animals/0.09 sq. m (1 sq. ft.). Plots were covered with conventional netting held down with rebar rods and staples after planting. The plots were maintained (with periodic net changes) by a Residential Americore volunteer working with Dr. Walton, as well as MBL interns, Roger Williams University students and P.I.s and were watched over by the Town of Barnstable shellfish department. Temperature was continuously monitored with Onset Continuous Temperature Monitors at each site. Additionally, Dr. Walton has deployed a YSI probe in Barnstable Harbor that had a direct web site link (it is deployed only during the summer) (http://www.ysieconet.com/public/WebUI/Default.aspx?hidCustomerID=88).

Because of the high initial mortality of Florida clams in the MBL facility upon receival from Florida, clams from a New Jersey source were also ordered and planted in 3 additional 2.13 x 2.13 m plots at both sites. 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 were not been fed after retrieval. They were used in the second set of laboratory experiments.

Starting in year two mortality was noted on the surface of plots so we began taking core samples from the plots (not in the original plan), core samples (30.5 cm in diameter) were taken from plots at each of the 3 sampling time periods of spring summer and fall (see objective 2 below). Live and dead were counted in each core sample and all animals were placed back in the plot. Nets were changed 2 times per year, usually at the time of sampling.

Unfortunately by the end of year two, clams in our control plots became positive for QPX infections. This necessitated acquiring additional animals from Florida and MA, instead of from our plots, in order to conduct the fourth laboratory study. However, at the beginning of the 4th laboratory experiment it was determined that the recently acquired, 2.5 cm shell height seed clams from our MA source were also positive for QPX infection. So, the 4th laboratory experiment was conducted with only Florida origin hard clams.

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 held recirculating units set at 2, 10, 12 and 14°C (this space was donated for no fee by the MBL). The additional recirculating units, set at 16, 18 and 21°C were installed within the MRC (this space is normally fee based, but the fee was partially waived for this project).

OBJECTIVE 1.a. No Stress

The first set of laboratory experiments began October 3, 2005. Six 30.5 x 30.5 cm plastic containers holding 2.5 cm 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 clam strain) in each tub. Animals were acclimated in the tubs at the correct temperature for 2 weeks before the experiment began. After two weeks, 100 of each strain of clam (FL and MA) were injected in the pericardial sac/adductor muscle sinus with 0.075 ml of QPX culture diluted with sea water and 50 of each type of clam were injected with 0.075ml of media diluted 1/10 with sterile sea water and clams were placed back into the appropriate containers. Samples were removed at 1, 2 and 3 months after injection. At sampling, animals were shucked, examined for gross abnormality, and fixed in 10% formalin in sea water and processed into paraffin sections as described later.

OBJECTIVE 1.b. and c. Stress caused by 4 months of low food availability and burrowing deprivation. and c. Acute, heat-induced stress.

For the second set of laboratory based experiments animals were collected from plots at Rendevous Point in the spring of 2006 (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 were injected and placed in the same containers as described above, however no sand was added to the containers and the 2 °C temperature

was not used. Animals were acclimated to the appropriate temperatures for 2 weeks before the experiment began. After two weeks, 80 of each strain of clam (FL and MA) were injected with 0.075 ml of QPX culture in a dilute media and 40 of each strain of calm were injected with 0.075 ml of media diluted 1/10 with sterile sea water and then placed back into the containers in the designated trays. Samples were removed at 1 and 2.5 months. A power outage at the MBL allowed the cold room to heat up to > 14° C over the weekend only a few days after the second sampling, so the P.I. decided to end this experiment. However, since the remaining animals appeared to be in good shape both in the cold room and in the MRC tanks, it was decided to use these injected animals to fulfill the 3rd laboratory objective. One tub of containers containing both strains of injected and control animals from the MRC and 2 tubs of containers from the cold room were selected for further manipulation. The temperature of the remaining originally designated 16° C tray in the MRC and the 14 and 12°C tubs in the cold room were varied from 14 to 20° and back on a three/four day cycle for one month. At the end of the month, the remaining clams were processed as described above.

OBJECTIVE 1.d. ADDITIONAL LAB EXPERIMENTS BASED ON PREVIOUS RESULTS

As described in the proposal, laboratory experiments attempted would be based on results from previous experiments so that logical next steps could be taken to understand temperature based pathogenic mechanisms. Before the next set of laboratory based experiments were conducted, Dr. Steven Roberts, a non-funded collaborator, showed that when QPX was cultured in sterile seawater with minced clams tissue, increased production of RNA occurred indicating increased proteases were being produced by QPX (in comparison to media cultured QPX). That information coupled with the lack of significant proliferative infections in the laboratory based clam experiments led to a new method for exposure of clams in the laboratory.

In the first of these additional experiments **(Laboratory Experiment 3)**, tubs were held at 16, 18 and 22° C. Clams of both MA and FL parentage, that has been deprived of food (some food was available through the water supply) and sand for one year, were placed in the containers and allowed to acclimate to the appropriate temperature for 2 weeks. Three treatments were applied to both strains of clam at all three temperatures (10 animals /strain/ treatment/ temperature). Treatments were: intra-sinusoidal injection of 0.1 ml of QPX cultured in minced clam tissue (QPX+TISSUE INJ), intra-sinusoidal injection of 0.1ml of QPX cultured in culture media (QPX + MEM INJ); and feeding via the water column in the containers of 5ml of QPX cultured in the minced clams tissue with sea water (QPX +TISSUE AQ). Injections occurred only once (after acclimation). Animals in designated containers were fed QPX two times per week for 5 weeks. All animals were shucked, examined grossly, fixed and processed for histological evaluation.

At the beginning of **Laboratory Experiment 4**, as described above, MA strains of hard clams were found to be infected with QPX, so only the Florida stain of clams were used. These clams and undergone no long term food depravation but did not have

sand (substrate) added to the containers during the experiment. Tub temperatures used in this experiment were 16, 18 and 21° C. This trial involved no injections, only adding different treatments to the water column in the containers. Treatments were: QPX cultured in sea water with minced clam tissue (20 animals at each temperature) (QPX + TISSUE AQ), QPX cultured in culture media (20 animals at each temperature) (QPX + MEM AQ) ; both of the previous methods with addition of 10 ml of cultured algae, T-Iso (20 animals at each temperature), and two controls (C) consisting of clams receiving no algae and clams receiving 10 ml of cultured T-Iso only (20 animals for each at each temperature) (previous designations +/- F) Animals were fed 2/3 times per week for 7 weeks after which all animals were sampled as described above.

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.

b. seven temperatures (with both QPX-infected and uninfected hard clams) in the laboratory temperature stress experiment.

Clams were collected from the plots and examined histologically seven times during the course of the experiment. At five of those sample periods, hemocyte evaluation was conducted on a subset of the samples (Hemocyte evaluation was not conducted on clams sampled from the first sample period of 11/05 and the last sample period of 10/07). All animals were collected from the plots in the harbor the day before processing. After retrieval, animals were quickly transported to temperature controlled containers where they were held till processing on the following day. The container temperature was matched with the current water temperature at the sample site. The processing was conducted at the Barnstable County Farmhouse (use donated to the project) with the help of Bill Walton, Barnstable County Extension Agent, and Dianne Murphy. During the project many people participated in processing these animals, In addition to Dr. Wikfors, and Helene Hégaret who conducted the analyses and Dr. Smolowitz and her technician, several graduate and visiting college students from U. CONN and the NOAA Milford Laboratory, student aquatic interns spending a semester at the MBL (from many different universities including Roger Williams University and Cape Cod Community College) and several Residential Americore representatives spent one to two days at each sample period processing clams. Between 400 and 100 clams were bled at each sample period to obtain hemolymph for flow cytometer evaluation (clams ranged form 2.5 cm shell height at the start of the field work to 5.3 cm in shell height at the end of the field work).

In addition to collection of hemolymph from each animal, measurements and weight were taken, animals were shucked and evaluated grossly and tissues for histopathological analysis were taken from 8 animals from each plot sampled at each time period. In addition to the testing of MA and FL clams as described in the grant, New Jersey origin clams were sampled at both the infected and control location at each time period. Wikfors and Hégaret brought the mobile flow cytometer to the county farm

house from the Milford laboratory at each sample period enabling them to analysis samples immediately. Target temperatures for collection were 13 °C for the spring and fall and 24 °C in the summer. However, because of the need to mobilized several people, the temperature targets were hard to hit in the spring and fall due to the quick changes in water temperature at those times of the year.

Due to the lack of proliferative infections in the laboratory based portion of the experiment, no hemocyte evaluation was conducted with these animals. However, an additional experiment was added to the study in which clams removed from infected plots were exposed to *Prorocentrum minimum*, a harmful dinoflagellate, which bloom recurrently and have been recorded on the East Coast of the USA. This phytoplankter has been reported to affect growth, survival, and organ and tissue development but also to affect hemocyte responses of bivalves.

Objectives of the additional experiment were to investigate possible interactions between this parasitic disease and exposure to *P. minimum* in *M. mercenaria*. Sixty quahogs with variable intensity of natural QPX infections were exposed, under controlled laboratory conditions to cultured *P. minimum* added to the natural, sympatric plankton at cell density equivalent to a natural bloom. After five days of exposure, clams were assessed individually for hemocyte parameters (morphological and functional, described below in "Immunological analysis") using flow-cytometry. Histological evaluation was also performed on individual clams to assess prevalence and intensity of parasitic infections, as well as other pathological conditions.

HEMOLYMPH STUDIES

Immunological Analysis:

Analyses of hemocyte morphology and function were done on hemolymph extracted from the clams. Hemolymph was withdrawn with a needle and a 1-ml syringe with 21 g needle from the adductor muscle of each individual clam, filtered with at 75 μm mesh, and stored temporarily in an Eppendorf microcentrifuge tube on ice to retard cell clumping. Hemolymph of up to six clams was pooled together for each analysis in first 3 sample periods. Animals were of sufficient size by the last 4 sample periods that hemolymph did not need to be pooled. Procedures for characterization of clam hemocytes and for function (mortality, phagocytosis, aggregation, apoptosis and oxidative burst) were adapted from Hégaret 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.

ADDITIONALLY EXPERIMENTS FUNDED BY THIS GRANT:
Gene Expression Analysis:

Because of the field study problems and the lack of laboratory induced disease, additional work was accomplished with the help of Dr. Roberts. In order to better understand the basic immune response and underlying differences across strains of hard clams faced with QPX, differentially expressed genes were identified. Two basic experiment were carried out using the GeneFishing DEG System including 1) comparing expression in putatively infected on non-infected Massachusetts clams and, 2) comparing expression across three strains of clams at different field sites. For both experiments, hemolymph was drawn into a 1 ml syringe from clams using a 21 g needle. Hemocytes were pelleted by centrifugation at 400g for 10 minutes. Supernatant was aspirated and discarded. Total RNA was isolated from hemocytes using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. RNA samples were treated with Turbo DNA-free (applied Biosystems/Ambion, Austin, TX) following manufacturer's protocol, to remove possible DNA carryover. This RNA was then used in the Genefishing DEG System as per manufacturer's instructions. Briefly, RNA was transcribed and a suite of random primers were used to perform PCR. The resulting PCR products were separated on an agarose gel, and differentially expressed bands removed and stored for DNA sequencing. Products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using an ABI 3730XL capillary sequencer (Applied Biosystems). All sequences were analyzed with NCBI Blast programs for similarity to known genes (Altschul, 1997). ClustalW (MacVector 7.2) analysis was used for sequence pair-wise and multiple protein alignments.

HISTOPATHOLOGICAL RATING OF FINDINGS:

Tissues from sampled clams (each clam was uniquely marked) were sampled by removal of sections of tissue as described in Smolowitz et. al., 1998, that included mantle, stomach, digestive gland foot, gonad, gill, heart, pericardial gland, and kidney. Sectioned were fixed in 10% formalin in sea water, processed in paraffin using standard methods. Histological sections (6 µm depth) were cut and stained with hematoxylin and eosin stains using standard methods (Humanson, 1979). These sections were evaluated for QPX. In order to evaluate tissue sections from both field and laboratory studies, a scheme of rating QPX occurrence and severity was developed. Histopathological sections form each animal examined was rated as follows (Fig. 1):

LOCATION OF LESIONS IN THE SECTION
Focal = 1
Multifocal = 2

Focally extensive = 3
Diffuse = 4

SEVERITY OF INFLAMMATION IN THE LESIONS (Fig. 1)
Mild = 1
Moderate = 2
Severe = 3

NUMBER OF VIABLE QPX ORGANISMS
None = 0
Less than 10 = 1
Between 10 and 50 = 2
More than 50 = 3

NUMBER OF DEAD ORGANISMS
None = 0
Less than 10 = 1
Between 10 and 50 = 2
More than 50 = 3

SIZE OF MUCUS HALOS AROUND TISSUE BOUND QPX
None = 0
Small rims (2-5 µm) = 1
Moderate rims (5 TO 15 µm) = 2
Large rims (greater the 15 µm) = 3

RESULTS AND DISCUSSION:
RESULTS OF LABORATORY EXPERIMENTS

PROLIFERATIVE VS. NON-PROLIFERATIVE LESIONS

Tissue sections from laboratory based experiments showed two types of lesions caused by QPX either when injected into the tissue or when added to the water column. These lesions were separated into proliferative and non-proliferative. When a QPX organism appeared in a tissue sections alone, and showed no to small halos, suggesting small amounts of mucus production, it was rated non-proliferative. If three or more organisms were identified side by side, in the same focus in a tissue section and/or were composed more than one tissue form of QPX and moderate to large amounts of mucus, the lesions was considered proliferative. (Figs. 2 and 3)

OBJECTIVE 1.a. No Stress

Examination of clams grossly showed no nodules or other proliferations upon shucking the animals. Histological examination of clams from all time periods showed no progressive infections in any animals. Clams from all time periods did show non-proliferative lesions in the tissues. The third, and last, sample period showed non-

proliferative lesions in several animals, but in no animals of either FL or MA stains held at 22° C (Fig. 4). More non-proliferative lesions were noted in the FL clams held at 14 °C and lower than in the MA clams. (All animals in the 2° C died before the experiment end. Histological evaluation indicated QPX was not the reason for their death). The non-proliferative lesions noted in injected animals are not considered true infections. However, they do show that the organisms can survive, although not proliferate, in the clam tissues when clams are held at lower temperatures.

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

Animals from laboratory experiment two were sampled twice to look for proliferative QPX infections. Shucked animals showed no nodules or other swellings when examined grossly. Histologically, non-proliferative QPX was identified in the tissues, but was not evaluated because these animals had been injected with QPX and the presence of QPX in the tissues did not indicate true proliferative infections (see Objective 1.a.). However, two animals in the second sample period did show proliferative lesions! Both animals were from the Florida clam strain. One animal was sampled from the 18° C tank and one animal, from the 12° C tank. These findings indicate true infections since the QPX organisms, although injected into the tissues, were proliferating (Fig. 5).

OBJECTIVE 1.c. Stress caused by 4 months of low food availability and burrowing deprivation. and acute, heat-induced stress.

One sampling period occurred following temperature variations as described for laboratory experiment two in the methods. Shucked animals showed no nodules or other swellings when examined grossly. Microscopically, one animal showed a proliferative infection (Fig. 6). This animal was a FL strain animal that had originally been held at 14°C before the temperature fluxuations began. This is considered a true infection. However, the percent of clams with proliferative infections was not greater than that seen in the first two sample periods before the temperature fluxuations began. This treatment did not cause an increase in proliferative infections in the laboratory exposed clams.

OBJECTIVE 1.d. ADDITIONAL LAB EXPERIMENTS BASED ON PREVIOUS RESULTS

LABORATORY EXPERIMENT 3

Animals were sampled and shucked. No nodules or swelling were noted grossly. Histopathologically, both non-proliferative and proliferative QPX infections were identified. Interestingly, non-proliferative lesions were more common and occurred at 3 temperatures of 16, 18 and 22° C in both FL and MA clams with the QPX + TISSUE INJ as compared to the QPX +MEM INJ clams. QPX + MEM INJ clams showed non-proliferative lesions only in clams held at 16° C clams (Fig. 7).

Proliferative QPX infections occurred in FL clams exposed to QPX+Tissue AQ (in the water column only) at both 18 and 16° C. **This is the first time that a natural infection has been accomplished in a laboratory exposure!**

One non-proliferative infection was identified in a FL clam that had been exposed to QPX + Tissue AQ. While this would not be considered significant if the QPX had been injected, it is considered significant in this case since infection while not proliferative had to have occurred via the water route and thus was a natural infection!

LABORATORY EXPERIMENT 4

Animals were sampled and shucked. No nodules or swelling were noted grossly. Histopathologically, a non-proliferative QPX infection was identified only in one FL clam held at 16° C and treated with QPX + TISSUE AQ - F (Fig. 8). While this would not be considered significant if the QPX had been injected, it is considered significant in this case since infection, while non-proliferative occurred via the water route and thus was a natural infection. The lack of other proliferative and non-proliferative infections does indicate that the clams may need to be stressed in some manner in order for infection to occur.

OBJECTIVE 2:

Quantify hemocyte types/morphologies and functional ability, including phagocytic ability, in the hemolymph of 2 strains of hard clams at three seasons/temperatures (spring, summer and fall) in both QPX-infected and uninfected clams cultured in the field.

Field Results:

A. Clams were sampled from field plot according to the following schedule and at temperatures noted:

5/6/2006	14.8° C
8/15/2006	21.5° C
10/13/2006	17° C
6/6/2007	16.7° C
8/1/2007	23° C
10/24/2007	17° C

B. Condition Index (C.I.) was accomplished using previously described methods (Smolowitz, et al., 1998; dry weight (g)/shell width (mm) x 1000) on subsets of clams in the fall of 2006 and 2007.
Results are as follows:

Fall 2006:
NJ = 26.71 average C.I.

MA = 26.34 average C.I.
FL = 34.35 average C.I.

Fall 2007:
MA = 47.90 average C.I..
NJ = 59.04 average C.I.
FL = no animals left in the plots

Comparison of Condition Index values shows that size appeared to have no effect on morbidity and mortality associated with QPX.

C. Mortality

At the end of the first winter, mortality was noted in all nine plots at both Scudders Lane and at Rendevous Point. Empty shells and boxes were lying on the surface of the sediment underneath the netting. Mortality was highest in the plots containing FL clams. Empty shells and boxes on the surface of each plot at Scudders Lane were counted. No QPX was noted histologically in the animals still retained in the plots at this time and mortality was attributed to over-wintering syndrome (Fig. 9).

Mortality with dead and dying clams at the sediment water interface was again identified in the Fall of 2006. Although not originally proposed as part of the study, plot core samples were added to the sampling methods for each time period from 10/2006 to 10 2007. Core sampled showed that mortality was most severe in the plots containing FL animals. However, mortality was more severe in MA than in NJ animals which contradict previously published reports (Ragone Calvo et al., 2007) (Fig. 10).

D. QPX infections and associated inflammation in field study clams

Twenty-five animals from each strain of clams from each location (Scudders Lane and Rendevous Point) were examined histologically at each time period for QPX (see rating scheme above). QPX infections were not detected in clam tissues until August, 2006 at Scudders Lane (Fig. 11). Unfortunately our control site clams at Rendevous Point became positive for QPX in the 10/06 sampling.

In addition to QPX prevalence, responding inflammation in the infected clams was rated (see above methods) (Fig. 12). Tallied results showed an interesting pattern when comparing QPX prevalence in clams from each sample period to average inflammation ratings at each sample period. When these results were compared a negative correlation between number of clams infected with QPX, and inflammatory response of the clam to QPX infection was identified. Such findings indicate that the lack of inflammatory response may have a permissive effect on the ability of QPX to proliferate in the clam tissues. Additionally the data suggest that FL strains of clams are less able to mount an inflammatory response in the spring and fall time periods of the first year after planting when compared to MA and NJ clams. Also, contrary to previous

findings, MA clam strains appeared more vulnerable to QPX infection than NJ strain of clams in this experiment.

E. Immunological Data. Effect of QPX Infections on Hemocyte Responses

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 origin of the population as well as the sites the clams are being grown in which could be related to the presence of QPX and intensity of infection.

We first assessed the effect of QPX on the clams as they were infected, by comparing hemocyte parameters of QPX-infected and non-infected quahogs. This comparison was only assessed for quahogs from Scudders Lane, for the three populations (MA, NJ and FL), in the spring and summer 2007, periods when the three populations of quahogs were infected by QPX. Indeed, both prevalence and intensity of QPX were much higher in quahogs located in Scudders Lane, than in clams from Rendevous point. In Rendevous point, quahogs did not become infected with QPX before June 2007. Conversely, in Scudders Lane, infection with QPX was first observed in clams from FL, followed by clams from MA, and finally clams from NJ in which QPX was only observed in June 2007. Thus, to assess the effect of QPX on the three populations, our attention was drawn to Scudders Lane, the most QPX-infected site.

Individual ANOVAs were run on each individual parameter, and no significant effect of QPX infection could be found. Thus, data from hemocyte parameters of the three populations of quahogs from Scudders Lane, in spring and summer 2007 were analyzed using Principal Components Analysis (PCA, Figure 13), followed by a One-Way ANOVA with infection by QPX as the independent variable and PCA Component 1 as the dependent variable. This approach was taken to determine if the combined hemocyte measurements could define a profile of QPX infection in quahogs. Our PCA included the data from hemocyte parameters of the three populations of quahogs from Scudders Lane, in spring and summer 2007.

The PCA plot (Figure 13) depicts correlations between the parameters according to the first component, which accounts for 44% of the variance in hemocyte characteristics. The following profile can be defined: high size and complexity of hemocytes, high percentage of dead or apoptotic hemocytes in the circulating hemolymph and low number of circulating hemocytes, and lower production of ROS. The ANOVA contrasting this profile with QPX infection was significant ($P < 0.01$, Figure 14). Thus, our results show that quahogs infected by QPX have a lower number of circulating hemocytes, a lower production of ROS, associated with a higher size and complexity of hemocytes, and a higher percentage of dead or apoptotic hemocytes, suggesting that quahogs infected by QPX have a weaker immune status. From this analysis, is was impossible to know whether QPX was causing this immuno-suppression or if QPX preferentially infects quahogs with a weaker immune status in the first place.

A second comparison of hemocyte parameters from the three populations (FL, MA, NJ), was assessed in quahogs from Scudders Lane before (October 2006) and after (June 2007) high early-spring mortality, probably attributable to QPX infection. Quahogs from the spring sampling had a higher prevalence of QPX (Fig. 11), but a much lower intensity of inflammation (Fig. 12). Quahogs also had a higher number of circulating hemocytes, and production of ROS by hemocytes, associated with a lower size and complexity of hemocytes in the spring 2007 than in the fall 2006, suggesting a stronger immune status of quahogs in June 2007, after the early spring, QPX mortality event. These results suggests that the weakest quahogs, still alive in October 2007 at Scudders Lane, had probably died, leaving preferentially quahogs with a stronger immune status (high number of circulating hemocytes, percentage of phagocytic hemocytes and production of ROS of hemocytes, and low size, complexity and percentage of dead hemocytes).

Another observation from this dataset was the intensity of hemocyte inflammation in the tissues, which increased significantly in quahogs during the summer of 2007 in quahogs from Scudders Lane. Simultaneously, a decrease in the prevalence of QPX was observed in the quahogs, despite the absence of mortality from spring 2007 to August 2007. Comparison of immune status of the three populations of quahogs in Spring and in the Summer of 2007 indicated a significant increase of the number of circulating hemocytes, percentage of phagocytic hemocytes and production of ROS by hemocytes, associated with a decrease of the size, complexity and percentage of apoptotic hemocytes (Figure 15), from Spring to Summer 2007. These results suggest a stronger immune status of the quahogs over the summer, which could also explain the decrease of intensity and prevalence of QPX infection in quahogs during the summer at high temperatures.

Together, all the results from this field study seem to indicate that quahogs with a stronger immune status, with stronger hemocyte responses (a high concentration of circulating hemocytes, probably newly formed, which would explain the smaller size and complexity of the hemocytes, associated with high percentage of phagocytosis, high production of reactive oxygen species, low percentage of dead or apoptotic hemocytes) were better prepared to overcome the infection by the parasite QPX.

Finally, the comparison between the three different populations at several sampling times indicates that, in October 2007, a significant difference was observed between the three populations of quahogs, with FL quahogs possessing a significantly-weaker immune status that NJ and MA quahogs (higher percentage of dead and apoptotic hemocytes, lower percentage of phagocytosis and production of ROS in hemocytes); whereas, after the mortality event, in spring and summer 2008, the differences in hemocyte parameters between the three populations were no longer significant. These results suggests selective survival of individuals with a stronger immune profile inform the initial population of FL quahogs not previously exposed to QPX. The FL clams suffered a massive mortality in early spring from QPX infection, whereas the other populations of quahogs NJ and MA, which have previously been exposed to QPX, had probably already been subjected to this selection.

F. ADDITIONAL EXPERIMENT: Possible interactions between the parasite QPX and exposure to the harmful dinoflagellate *PROROCENTRUM MINIMUM*

Objectives of the additional experiment were to investigate combined effects of two stressors. Quahogs with variable intensity of infection with QPX were exposed, under controlled laboratory conditions, to cultured *P. minimum* added to the natural, sympatric plankton at a cell density equivalent to a natural bloom.

Exposure of quahogs to *P. minimum* resulted in: a lower percentage of phagocytic hemocytes, higher production of reactive oxygen species (ROS), larger hemocyte size, more-numerous hemocytic aggregates, and increased numbers of hemocytes in gills accompanied by vacuolation and hyperplasia of the water-tubular epithelial cells of the gills. Quahogs sampled had a low prevalence of QPX; by chance, the parasite was present only in quahogs exposed to *P. minimum*. Thus, the effect of QPX alone on the hemocyte parameters of quahogs could not be assessed in this experiment, but it was possible to assess different responses of infected *versus* non-infected quahogs to *P. minimum*.

QPX infection was most often observed in the mantle at the base of the siphon, where multifocal and focally-extensive QPX infection associated with intense hemocytic infiltration was observed. Most QPX cells observed in these infiltrated areas of the mantle were dead and were observed as dark-pink stippling within light-pink, round cells having indistinct or poorly-distinguished cell walls surrounded by hemocytes or found within the cytoplasm of hemocytes. A few clams showed QPX infections accompanied by hemocytic infiltrations within the gills and connective tissues around the digestive tubules. Such findings of infections in the connective tissues surrounding the digestive tubules and in sinusoids of the gills, in addition to the mantle, usually indicate an overall more-extensive infection of the clam, as compared to infection in the mantle alone. Histological sections revealed that effects of QPX included intense hemocytic infiltration into the connective tissues of several organs including mantle and gills.

In addition, quahogs exposed to *P. minimum* and infected with QPX showed a mild increase in hemocytes within the gills. Thus, animals both infected with QPX and exposed to the HAB had the highest hemocytic infiltration, indicating combined effects of both stressors. Results also demonstrate that infection with QPX in quahogs exposed to *P. minimum* had smaller hemocytes and increasing hemocyte infiltration throughout the soft tissues. One hypothesis explaining the decrease in the size of hemocytes could be that new, smaller hemocytes were produced in response to the increasing movement of hemocytes toward the tissues infected with QPX, as observed by the increase of hemocytic infiltration surrounding the parasites.

This study shows that QPX disease in quahog clams can affect the response and the resistance of quahogs to environmental stressors, such as harmful algal blooms, highlights the importance of considering synergistic effects of different factors on the immunology and histopathology of bivalve shellfish, and demonstrates that parasites can affect the sensitivity of bivalves to harmful algae.

G. Gene Expression Analysis

Gene Expression Analysis: Experiment 1

For the first experiment carried out to identify differentially expressed genes QPX infected hard clams, RNA from one strain of Massachusetts clams were used with RNA pooled from six putatively infected and six putatively non-infected clams used for analysis. Five random primer sets produced approximately 10 products that were expressed at different levels in hemocytes from infected and non-infected clams. Fifty percent of the products were upregulated in infected clams and fifty percent were downregulated. In total approximately 45 clones were sequenced in both directions. Interestingly, while the sequence quality was good only about half of the sequence produced a hit using BLAST. Of those sequences the average sequence length was 502 bp. Only two results have e-values greater than 1.00E-05, a product similar to a serine protease inhibitor and a product similar to a vitelline membrane outer layer protein. Both of these products were upregulated in hemocytes harvested from QPX infected clams.

Gene Expression Analysis: Experiment 2

For the second DEG experiment hemocytes were used that were taken from clam strains originating from three locales; Massachusetts, New Jersey and Florida. As described elsewhere these three strains were planted in plots in Massachusetts where QPX is prevalent and clearly show different susceptibility to disease. Pooled samples were compared for clams in the same plots. Fig. 16 shows a representative gel. For this experiment a total of twenty bands were removed. Eight bands were upregulated in New Jersey hemocytes, 11 from Florida hemocytes and one from Massachusetts. These bands were cloned and with multiple product sizes indentified following cloning and sequencing in two directions, 192 sequencing reactions were carried out. As was the case for the first experiment, numerous products could not be identified based on sequence comparisons (BLAST). Below are listed some of the genes that were putatively identified

based on sequence similarity and the strain of clams where the given product was determined to be upregulated.

Gene	**Clam Strain upregulated in**
disease resistance RPP5 like protein	New Jersey
variable lymphocyte receptor diversity region	Massachusetts
ribosomal protein L22e	Florida
ribosomal protein L23A	New Jersey
ribosomal protein rps3	New Jersey
hypothetical protein Cag_0759	Florida
ATP synthase subunit 6	Florida
NADH dehydrogenase subunit 6	Florida
60S ribosomal protein L37	Florida
myosin heavy chain	Massachusetts

CONCLUSIONS:

LABORATORY WORK:

Infection of non-stressed hard clams (laboratory experiment 1) by injecting QPX cultured in standard culture media did not produce proliferative infections. Non-proliferative lesions were noted in clams held at lower temperatures (vs. 22°C) which indicates that clams held at 22° C were more able to kill and remove the injected QPX organisms thus indicating a better operational inflammatory system in clams held at higher temperatures. Additionally, FL clams showed more non-proliferative infections that MA clams which again suggests less ability of the inflammatory system of FL clams to react to an insult such as QPX infection at lower temperatures.

When clams (laboratory experiment 2) were stressed (no food and no substrate), infections resulting from injection of QPX cultured in standard culture into the pericardial sac/adductor muscle sinus did produce some proliferative lesions in FL clams held at temperatures of 18° C and less. These findings again indicating that stress, temperature and strain were important in the production of QPX disease.

The third laboratory experiment, in which severely stressed clams were injected with QPX cultured in either standard media, or sea water with minced clam tissues, showed QPX cultured in sea water with minced clam tissues is more able to resist a clam's inflammatory response. Again, the FL stain of clams showed more non-proliferative infections than the MA strain of clams. Clams exposed at lower temperatures showed more non-proliferative lesions than clams held at 22° C. These results again show that lower temperatures, stress and strain of clam were important in the effectiveness of the inflammatory response.

The third experiment was able to produce two proliferative lesions and one non-proliferative lesion in FL clams held at 18 and 16 °C by water column exposure of the clam to QPX cultured in sea water with minced clams. These infections all occurred in the mantle at the base of the siphon. This is the first time that infection by water column exposure has been accomplished! It indicates clam stress, lower temperature of the water, clam strain and **heightened QPX infective abilities** are all important in the production of disease.

The fourth experiment, in which mildly stressed FL clams were exposed to QPX cultured with minced tissue in sea water, showed that non-proliferative infections could occur in mildly-stressed, strain susceptible clams with water column exposure.

In general, while the proliferative infections produced in this study were not in high numbers, the results do allow use to make the following conclusions:

a. The FL clam strain was less resistant to QPX at lower temperatures (18, 16 and 14°C) than the MA stain indicating FL clams have a less responsive inflammatory system at lower temperatures.

b. QPX grown on minced clam tissue was more infectious than QPX grown in standard media. This suggests that if environmental QPX is exposed to dead and dying clam tissues, it may become more pathogenic to clams thus promoting QPX disease in an area where disease was not seen before.

c. Proliferative infections could be produced by exposure of stressed, susceptible clams to QPX added to the water column indicating stress and clam strain are important in development of the disease. Overwintering stress might play a role in this disease.

d. The base of the siphon/adjacent mantle is the most vulnerable location to QPX infection. Pseudofeces is held in this area for varying periods of time. Activated QPX, if part of a pseudofeces pellet, may have a longer period of exposure to adjacent tissues allowing for infection.

FIELD WORK:

Several conclusions can be drawn from the field work accomplished in this study. First, the study showed (as in the lab) that FL clam strains were much more susceptible to QPX infection than MA or NJ clam strains. However, in our study, unlike a previous study (Ragone-Calvo et al., 2007), MA animals were more susceptible to disease than NJ clam strains. There are numerous theories why this might have happened, but the conclusion that can be drawn is that there is higher variability that previously thought between clam strains in relationship to geographical origin and the clams ability to avoid QPX caused morbidity. Immunological and genetic evaluations of clams are needed to identify what the important components of resistance are in a strain of clams.

This experiment provides a better understanding of the role of hemocytes and immune status in QPX resistance of quahogs. The results of this study highlight the importance of the immune status in the survival of Quahogs when challenged by QPX. The study demonstrates that QPX-infected quahogs have a weaker immune status (lower number of circulating hemocytes, lower production of ROS, associated with higher size and complexity of hemocytes, and a higher percentage of dead or apoptotic hemocytes). These findings are very important for aquaculture purposes as they may be used to better select the quahogs that will be grown in regions where QPX occurs and possibly reduce the negative impact of QPX on the aquaculture of quahogs.

Indeed, an important finding of this experiment is the suggestion that QPX causes the death of the weakest individuals in a population, leaving the quahogs with a stronger immune profile alive. This hypothesis is re-enforced by the fact that FL quahogs which had never been exposed to QPX, were the most sensitive and died very early in the experiment from QPX disease. This study also showed that an existing QPX infection

can affect the response and resistance of quahogs to a HAB. This suggests that the susceptibility to both stresses may be related and that highly-parasitized bivalves can be more susceptible to HAB effects, and probably to other types of environmental stresses as well.

An additional benefit of this work was the ability of many students to participate in the study, including learning how the flow cytometer could be used to study bivalve processes (Fig. 17).

Gene Expression Analysis work identified two gene products that were up-regulated in QPX infected clams in the first experiment. The second set of experiments identified genes that were up-regulated in the three different clam strains. These findings are novel and while exact gene bank matches and definite assignment of function of these genes cannot be determined at this time, they are part of the gene discovery pathway that will eventually lead to understanding the inflammatory response of clams to QPX (and probably other) diseases. They will eventually provide the means for identification of disease resistant brood stock. Dr. Roberts will continue this work in another grant funded by NRAC titled "Evaluation of putatively QPX - resistant strains of northern hard clams using field and genetic studies".

IMPACTS:

Laboratory Infection Trials:

Stress and brood stock origin both showed significant positive effects on the occurrence of QPX disease in hard clams. Importantly, previous adaption of QPX to growth on clam tissue in sea water in culture, vs. growth in culture media alone, appeared to increase the likely-hood of infection of naive clams. These finding suggest reducing mortality from other causes in a plot of cultured clams would decrease the likely-hood of QPX infection in that plot.

Field Work Results:

Results of this experiment show that Florida clams are more sensitive to QPX disease that clams originating from brood stock acclimated to more northern climates. However, results of this study did slightly contradict previous work (Ragone-Calvo et al., 2007). In our study, clams from New Jersey appeared to survive better than clams from Massachusetts (the most northern brood stock location). Such findings indicate that restriction to importation of seed clams based on state origin alone may not be totally effective.

The immunological results of this study highlight the importance of the immune status in the survival of Quahogs when challenged by QPX. The study demonstrates that QPX-infected quahogs have a weaker immune status and suggests these parameters may

be measured to better select the quahogs that will be grown in regions where QPX occurs and possibly reduce the negative impact of QPX on the aquaculture of quahogs.

Indeed, an important finding of this experiment is the suggestion that QPX causes the death of the weakest individuals in a population, leaving the quahogs with a stronger immune profile alive. Suggesting that development of resistance is possible. The study also showed that an existing QPX infection can affect the response and resistance of quahogs to a HAB. This suggests that the susceptibility to both stresses may be related and that highly-parasitized bivalves can be more susceptible to HAB effects, and probably to other types of environmental stresses as well.

This study has laid the ground work for evaluation of gene expression in relationship to clam strain and susceptibility to QPX disease. Understanding differences and developing ability to identify these in brood stock will provide new tools for the aquaculture industry. Work on this subject will continue as part of a newly funded NRAC study entitled "Evaluation of putatively QPX - resistant strains of northern hard clams using field and genetic studies".

SUPPORT:

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT UNIVERSITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL TOTAL SUPPORT
5/2005 TO 7/15/08	$154,805 (TWO YEAR TOTAL AWARDED)					$154,805
5/2005 TO 7/15/0					$6615* $24,111** $3290***	$34,016

*This is the approximate worth of the time and materials donated by Barnstable County to the project during the course of the study. The use of the Barnstable County farm house was offered for free to this project. Dr. William Walton (and Dianne Murphy, his assistant) have donated time to this project as stated in the grant.
**This amount reflects the space, holding trays and water supply support that has been donated by the MBL to this project (a dedicated cold room, two A-frames and recirculating unit shelf space in a mariculture room at the MRC and supplies of both flowing ambient and treated sea water.
*** Total worth of free rooming provided for researchers by Coonamessett Farm during field sampling periods.

Additional Support/Complementary Research

Another study funded by NSF/EID interdigitated with this work. A study funded to E. Ward (U. of Conn.), on which Dr. Smolowitz is a co-PI, is examining, as a part of that grant, the occurrence of QPX in "marine snow" over Smolowitz's positive and negative samples sites over the next two years. This data will be integrated with data gathered as part of the NRAC grant to help develop an idea of occurrence of QPX in the water column and in marine snow above the sites (and thus information concerning how infection occurs between clams and sites) vs. the occurrence of QPX in the clams (infected site) or no disease in the clams (control site). This work will is using a real time PCR method that has been developed in the laboratory of Steven Roberts at the MBL as a method for measurement of QPX in the snow, water and sediment in and over the plots.

APPENDIX 1.

PAPERS PRESENTED:

1. Smolowitz, R. Barnstable County QPX Aquaculture Meetings.
a. Wellfleet, MA (May 2005)
b. Courthouse, Town of Barnstable, Barnstable, MA (May, 2005).

At these meetings (the idea for which originated from Smolowitz) researchers met with town shellfish wardens, county representative and culturists to explain ongoing (and planned) research for areas affected by QPX.

2. Smolowitz, R. A review of QPX disease with an emphasis on initiation and progression in wild and cultured populations of Mercenaria mercenaria. Monterey, CA, National Shellfish Association Conference, 2006.

3. Hégaret, H., Smolowitz, R.M., Wikfors, G.H., DeFaveri, J., Walton, W., Murphy, D., Shumway, S.E. Effect of temperature on hemocyte responses of northern quahogs (=hard clams, *Mercenaria mercenaria*) from different populations, infected by the protistan organism, Quahog Parasite Unknown. Providence, RI, National Shellfish Association Conference, 2008.

4. DeFaveri, J., Roberts, S., Smolowitz, R., Murphy, D., Walton, W. Characterizing gene expression patterns in three strains of northern quahogs Mercenaria mercenaria in response to infection by QPX. . Providence, RI, National Shellfish Association Conference, 2008.

5. Smolowitz, R., DeFaveri, J., Walton, W., Murphy, D., Leavitt, D. The interaction of temperature and hard clam (Mercenaria mercenaria) strain on the occurrence of QPX disease in the laboratory. Providence, RI, National Shellfish Association Conference, 2008.

6. Hégaret, H., Wikfors, G.H., Smolowitz, R., Shumway, S. E. Effect of the harmful algal *Prorocentrum minimum* on the hemocyte response of quahogs *Mercenaria mercenaria* with various levels of QPX infection. Woods Hole, MA, Fourth Symposium on Harmful Algae in the U.S. November, 2007.

7. Smolowitz, R. Recent knowledge concerning QPX infections in hard clams. Buford, SC., 33rd Eastern Fish Health Conference, S.C., April 2008.

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Hégaret, H., Wikfors, G.H., Soudant, P., 2003b. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. Journal of Experimental Marine Biology and Ecology 293, 249-265.

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Lambert, C., P. Soudant, G. Choquet and C. Paillard. 2003. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish and Shellfish Immunology 15: 225-240.

Ragone Calvo, L., S. Ford, J. Kraeuter, D. Leavitt, R. Smolowitz and E. Burreson. 2007. Influence of host genetic origin nad geographic location on QPX disease in northern quahogs (= hard clams), *Mercenaria mercenaria*. Journal of Shellfish Research: 109-110.

Smolowitz, R., D. Leavitt and F. Perkins. 1998. Observations of a Protistan disease similar to QPX in Mercenaria mercenaria (hard clams) from the coast of Massachusetts. Journal of Invertebrate Pathology 71: 9-25.

FIGURE LEGEND

Figure 1. Photomicrographs of clam sections representing different inflammatory severity ratings used to evaluate histological sections. 1.A. A focus of QPX infection with an inflammatory response rating of 1. 1.B. A focus with a rating of 1.5. 1.C. A focus with a rating of 3. (Photomicrographs taken from 6 μm sections of paraffin embedded clam tissue stained with hematoxylin and eosin.)

Figure 2. A. and B. Examples of non-proliferative lesions as identified histologically. (Photomicrographs taken from 6 μm sections of paraffin embedded clam tissue stained with hematoxylin and eosin.)

Figure 3. A., B. and C. Examples of proliferative lesions as identified histologically. (Photomicrographs taken from 6 μm sections of paraffin embedded clam tissue stained with hematoxylin and eosin.)

Figure 4. Percent of clams from experiment 1, the 3rd sample period, that contain non-proliferative lesions in the tissues as identified histopathologically.

Figure 5. Percent of clams with proliferative QPX infections from the second sample period of laboratory experiment 2.

Figure 6. Percent of clams with proliferative lesions after temperature stress exposure.

Figure 7. Percent of clams with both proliferative and non-proliferative QPX lesions from laboratory experiment 3.

Figure 8. Percent of clams with proliferative QPX lesions from laboratory experiment 4.

Figure 9. Over-wintering mortality. Total number of boxes on the sediment surface for each strain of clam (3 plots/strain) in spring 2006.

Figure 10. Percent mortality of each strain of clam (cumulative total of all three plots/strain) at the fall sampling at Scudders Lane.

Figure 11. Percent of all clam strains from Scudders Lane infected with QPX at each sample period.

Figure 12. Average inflammation index for each clam strain infected with QPX at each sample period from Scudders Lane.

Figure 13: Principal Components Analysis plot of selected hemocyte parameters from quahogs located in Scudders Lane in June and August 2007.

Figure 14: Plot of effect of infection of QPX, with Component 1 from Principal Components Analysis of hemocyte parameters from quahogs located in Scudders Lane in June and August 2007, as dependant variable (ANOVA $P < 0.01$).

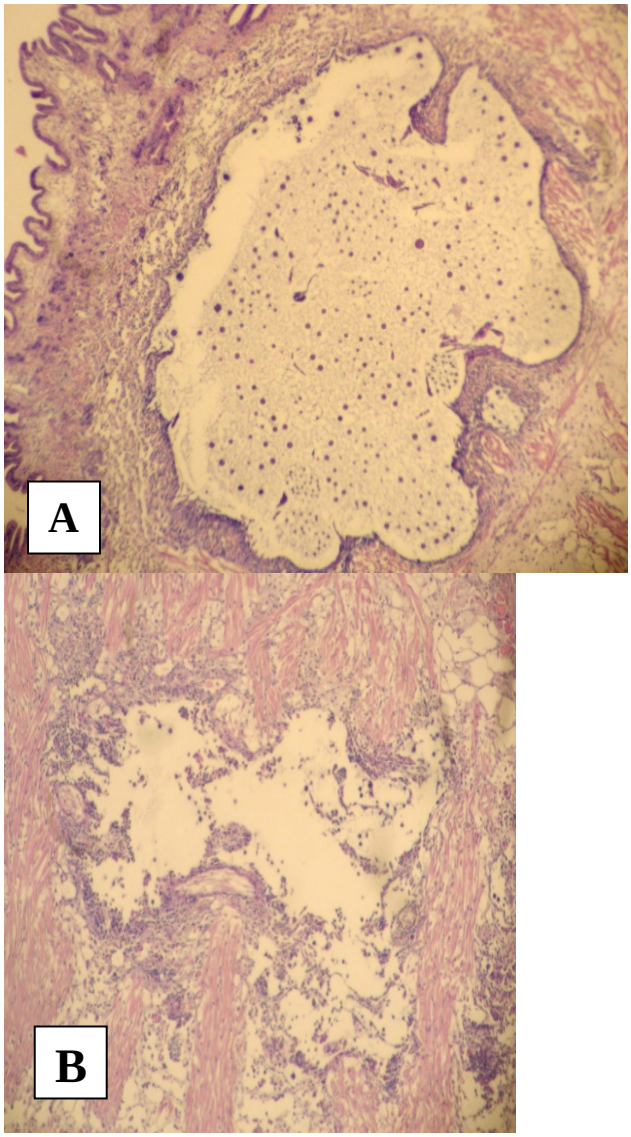
Figure 15: Differences in hemocyte parameters of quahogs from the three populations from spring (Jun. 07) and summer (Aug. 07) (Scudders Lane only shown).

Figure 16. Partial deduced amino acid sequence alignment of hard clam serine protease inhibitor gene (top) and a *Serine protease inhibitor dipetalogastin precursor* from the arthropod *D. maximus* (GenBank # O96790). Horizontal red line indicates domain that inhibits subtilisin-like serine proteases and asterisks indicated conserved cysteines required for tertiary structure.

Figure 17. Image of agarose gel where primers pairs (52-57) were used to amplify genes expressed in hemocytes from infected clams from three strains; Massachusetts, New Jersey and Florida. For each set (between vertical lines) the sample order is Massachusetts, New Jersey and Florida. Circles indicate bands that were excised and sequenced.

Figure 18. Dr. Wikfors demonstrating the use of the FACS to clam bleeders at the Barnstable Farm house.

Figure 1.



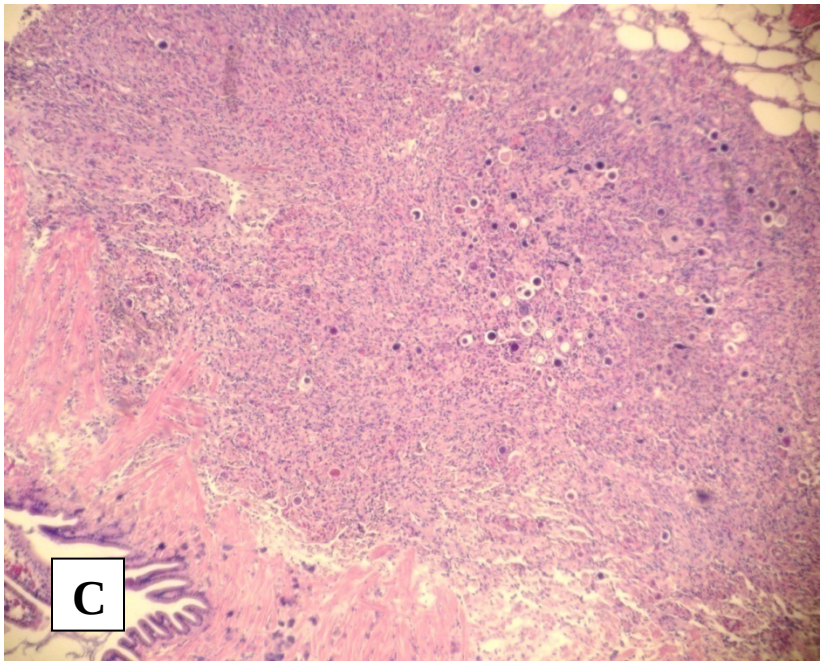

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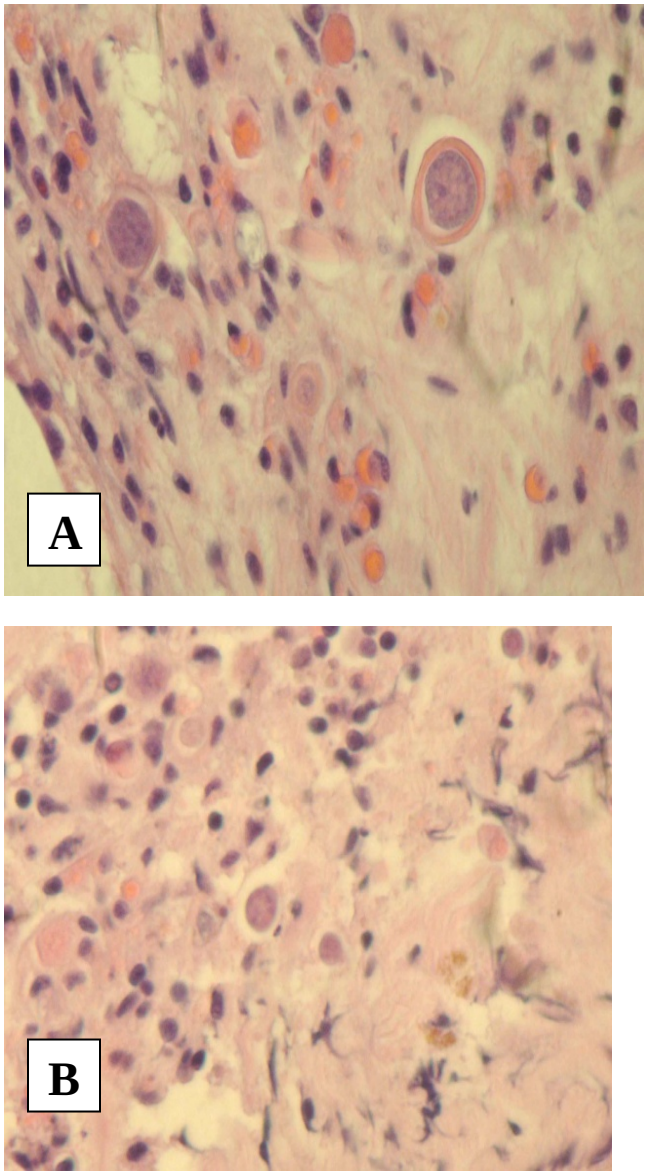
Figure 2.



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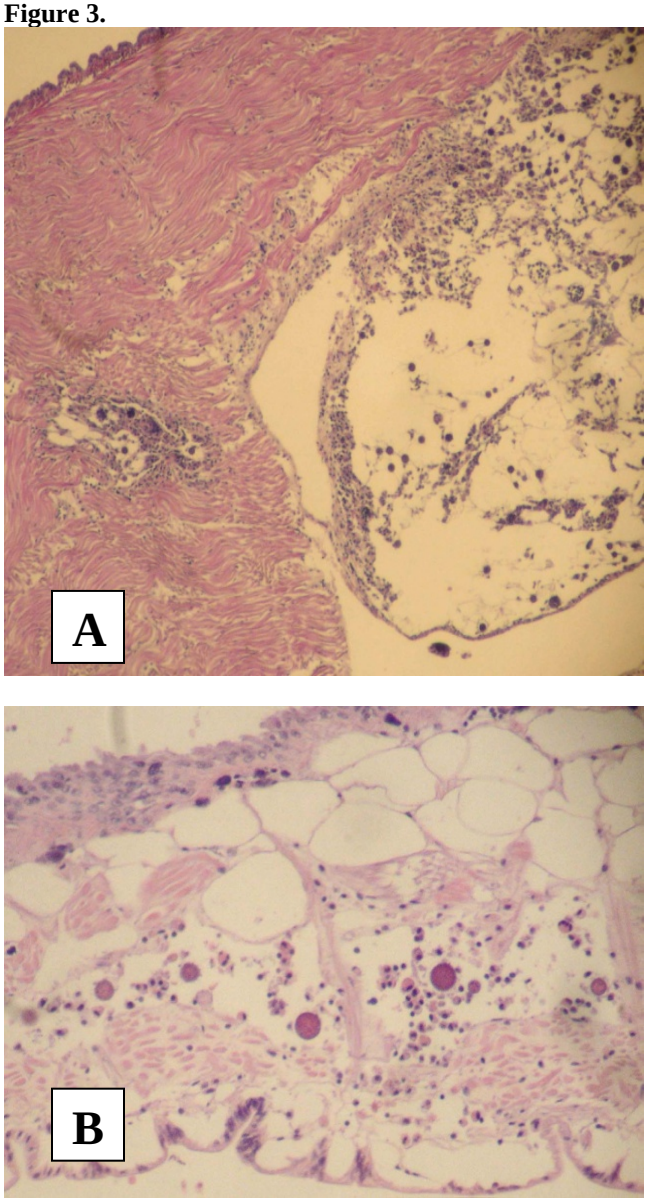

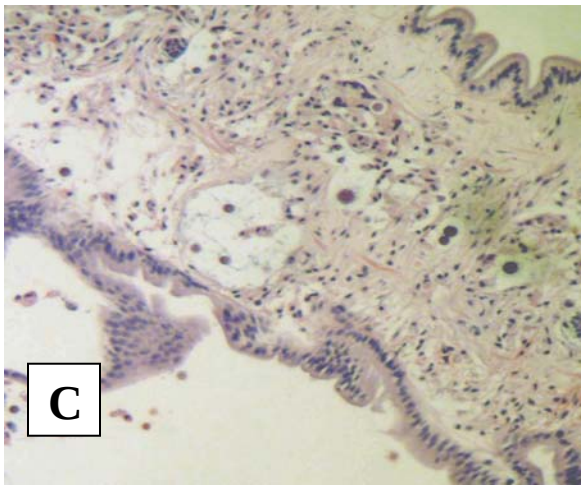
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Figure 4.

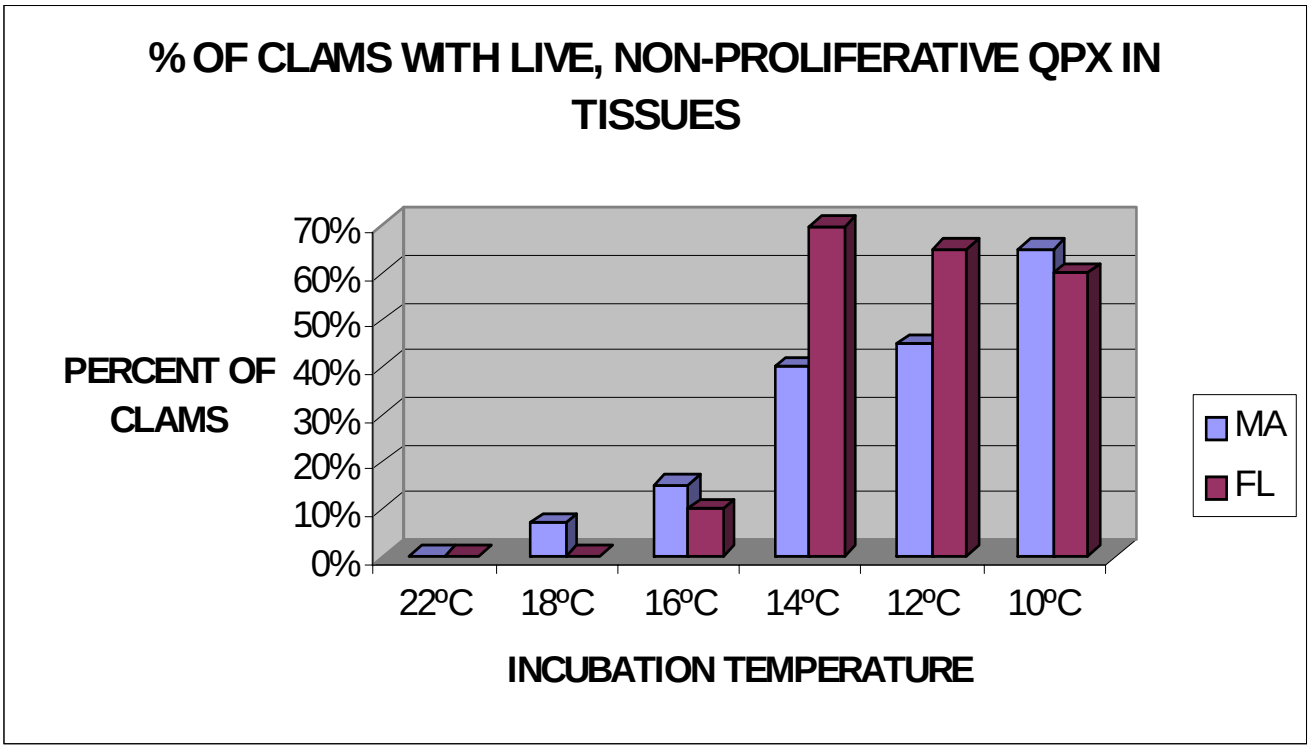
% OF CLAMS WITH LIVE, NON-PROLIFERATIVE QPX IN TISSUES
PERCENT OF CLAMS
70%
60%
50%
40%
30%
20%
10%
0%
22ºC
18ºC
16ºC
14ºC
12ºC
10ºC
INCUBATION TEMPERATURE
MA
FL

Figure 5.

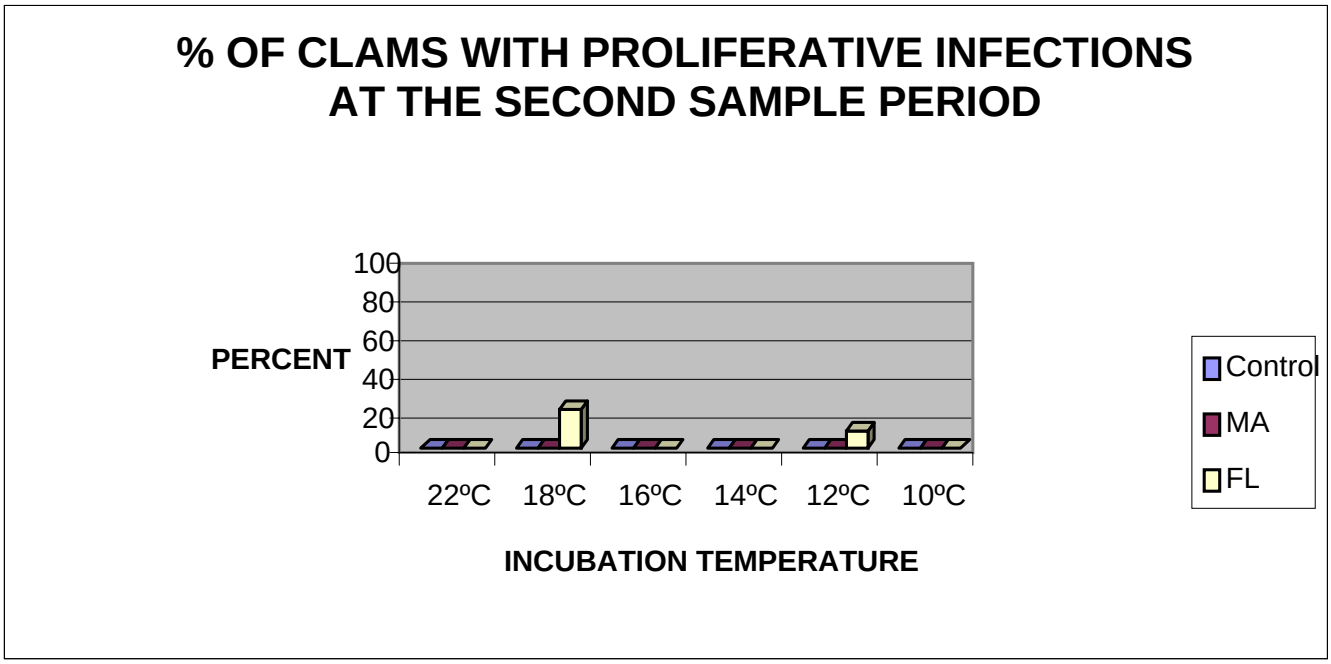
% OF CLAMS WITH PROLIFERATIVE INFECTIONS AT THE SECOND SAMPLE PERIOD
PERCENT
100
80
60
40
20
0
22ºC
18ºC
16ºC
14ºC
12ºC
10ºC
INCUBATION TEMPERATURE
Control
MA
FL

Figure 6.

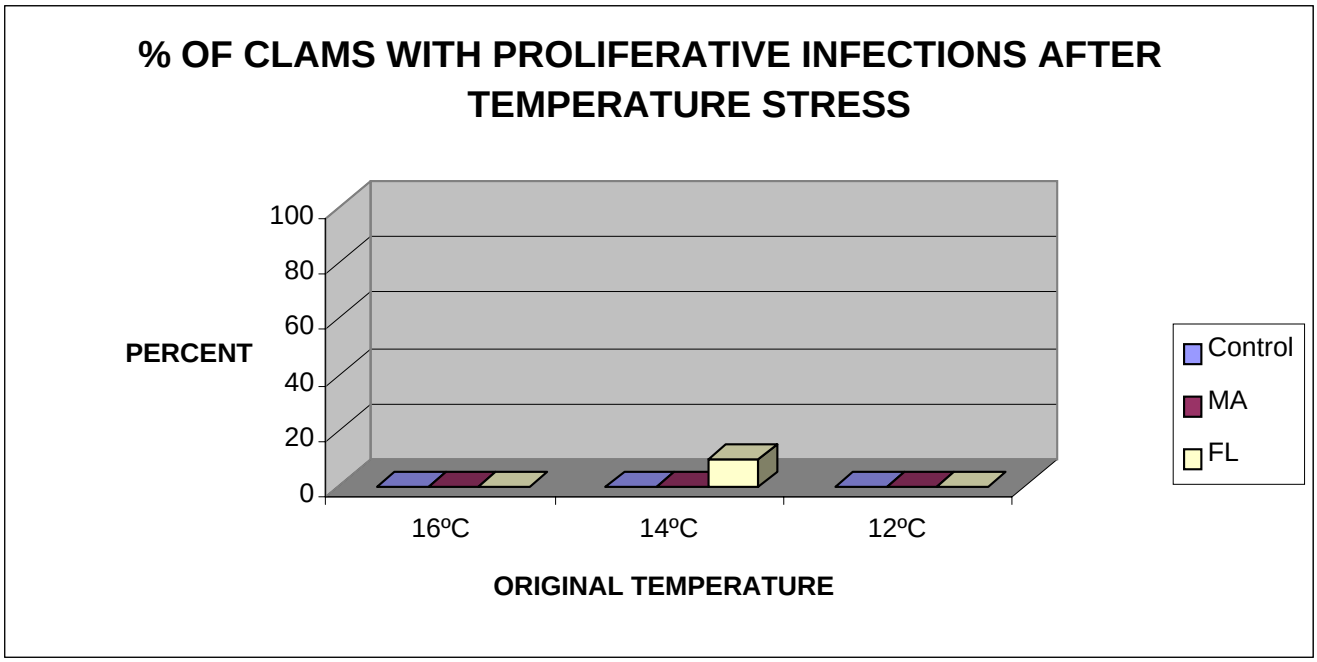
% OF CLAMS WITH PROLIFERATIVE INFECTIONS AFTER TEMPERATURE STRESS
PERCENT
100
80
60
40
20
0
16ºC
14ºC
12ºC
ORIGINAL TEMPERATURE
Control
MA
FL

Figure 7.

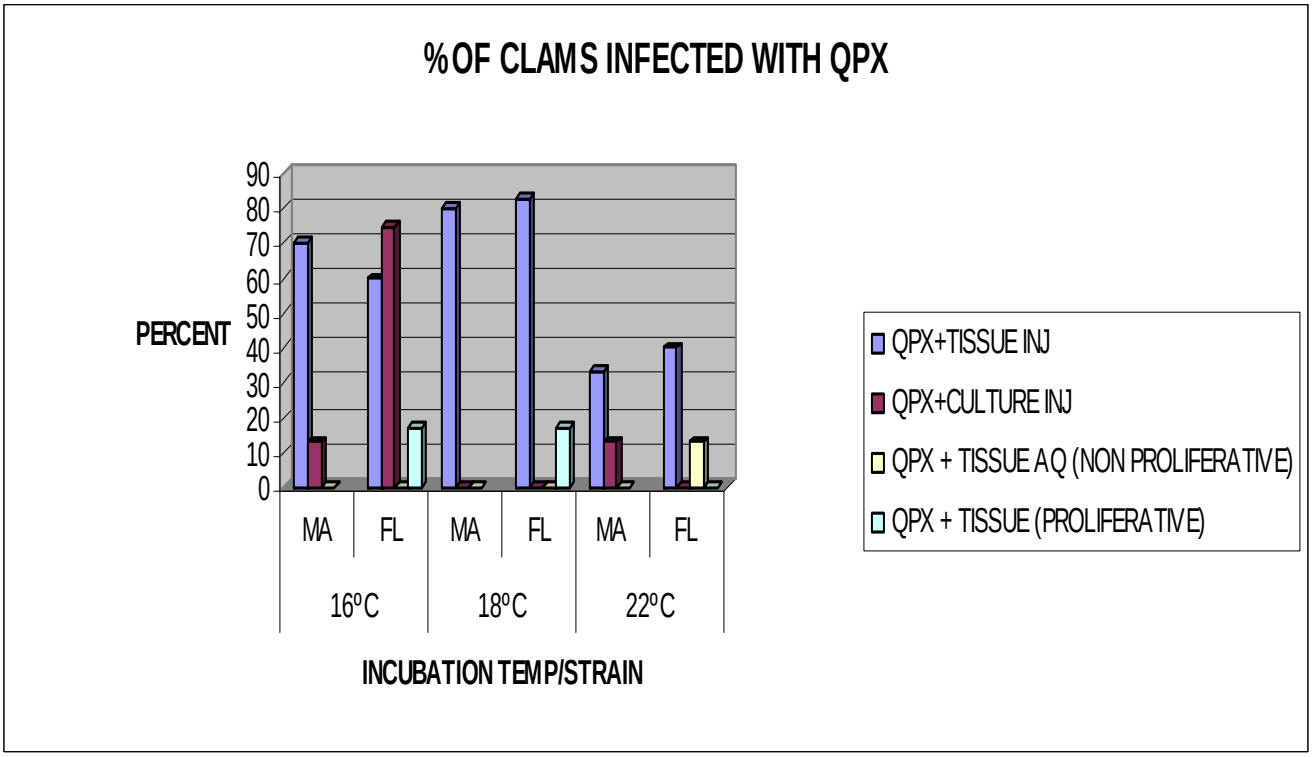
%OF CLAMS INFECTED WITH QPX
PERCENT
90
80
70
60
50
40
30
20
10
0
MA
FL
MA
FL
MA
FL
16°C
18°C
22°C
INCUBATION TEMP/STRAIN
QPX+TISSUE INJ
QPX+CULTURE INJ
QPX + TISSUE AQ (NON PROLIFERATIVE)
QPX + TISSUE (PROLIFERATIVE)

Figure 8.

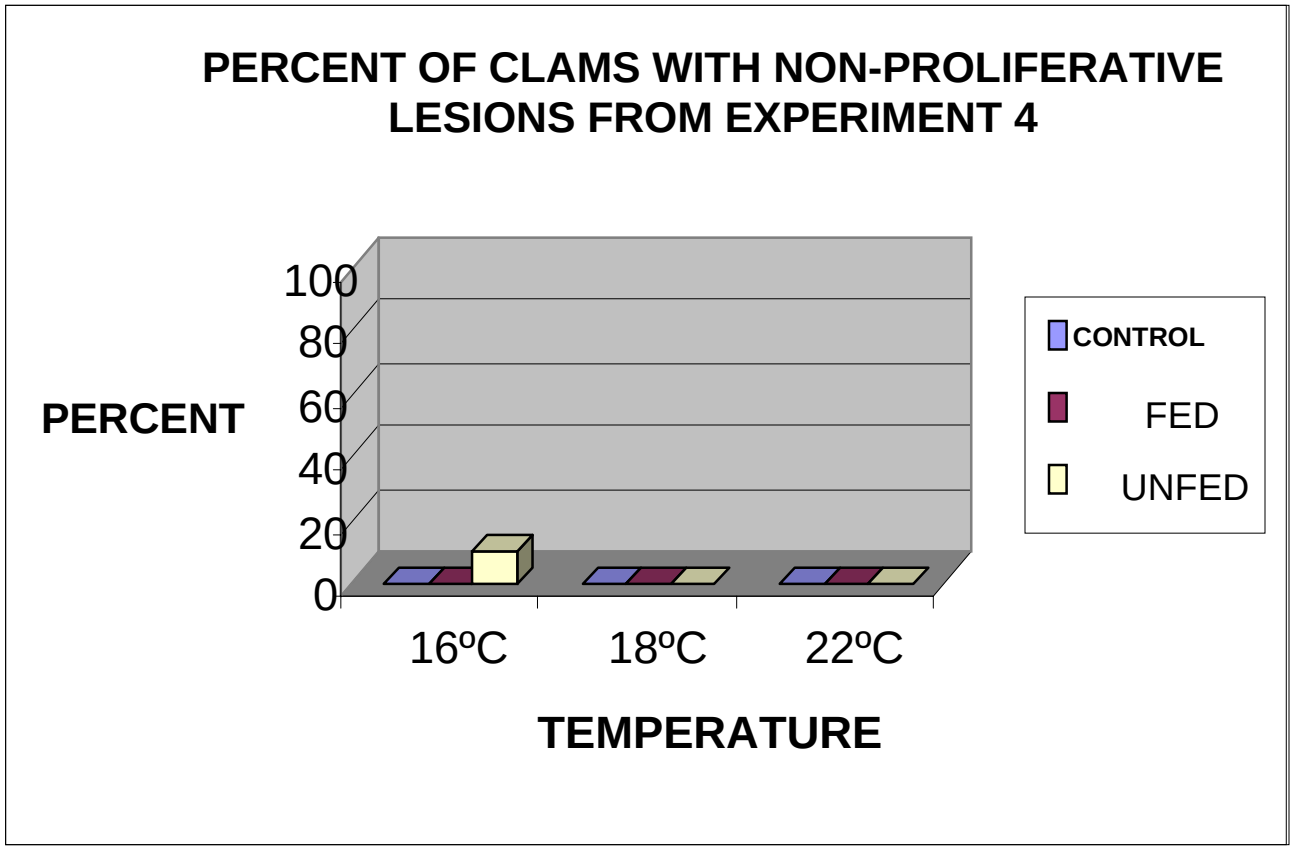
PERCENT OF CLAMS WITH NON-PROLIFERATIVE LESIONS FROM EXPERIMENT 4
PERCENT
100
80
60
40
20
0
16ºC
18ºC
22ºC
TEMPERATURE
CONTROL
FED
UNFED

Figure 9.

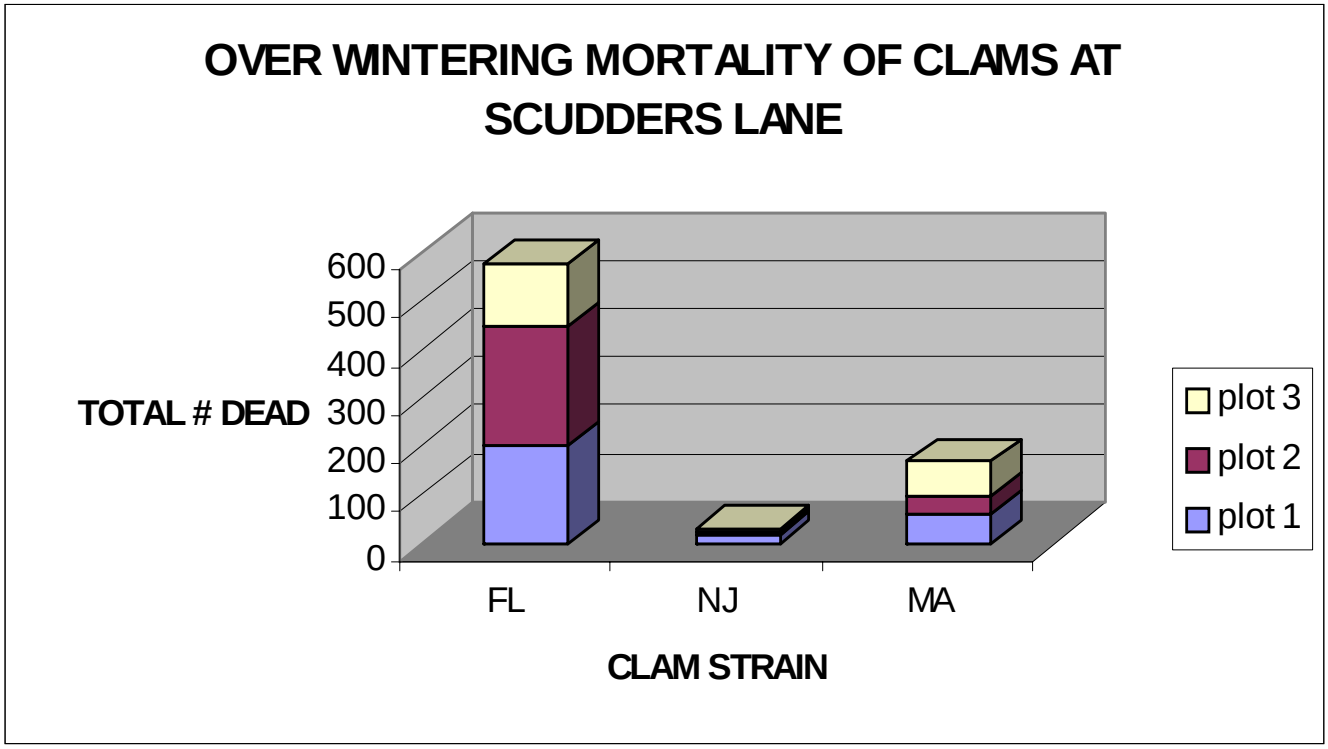
OVER WINTERING MORTALITY OF CLAMS AT SCUDDERS LANE
TOTAL # DEAD
600
500
400
300
200
100
0
FL
NJ
MA
CLAM STRAIN
plot 3
plot 2
plot 1

Figure 10.

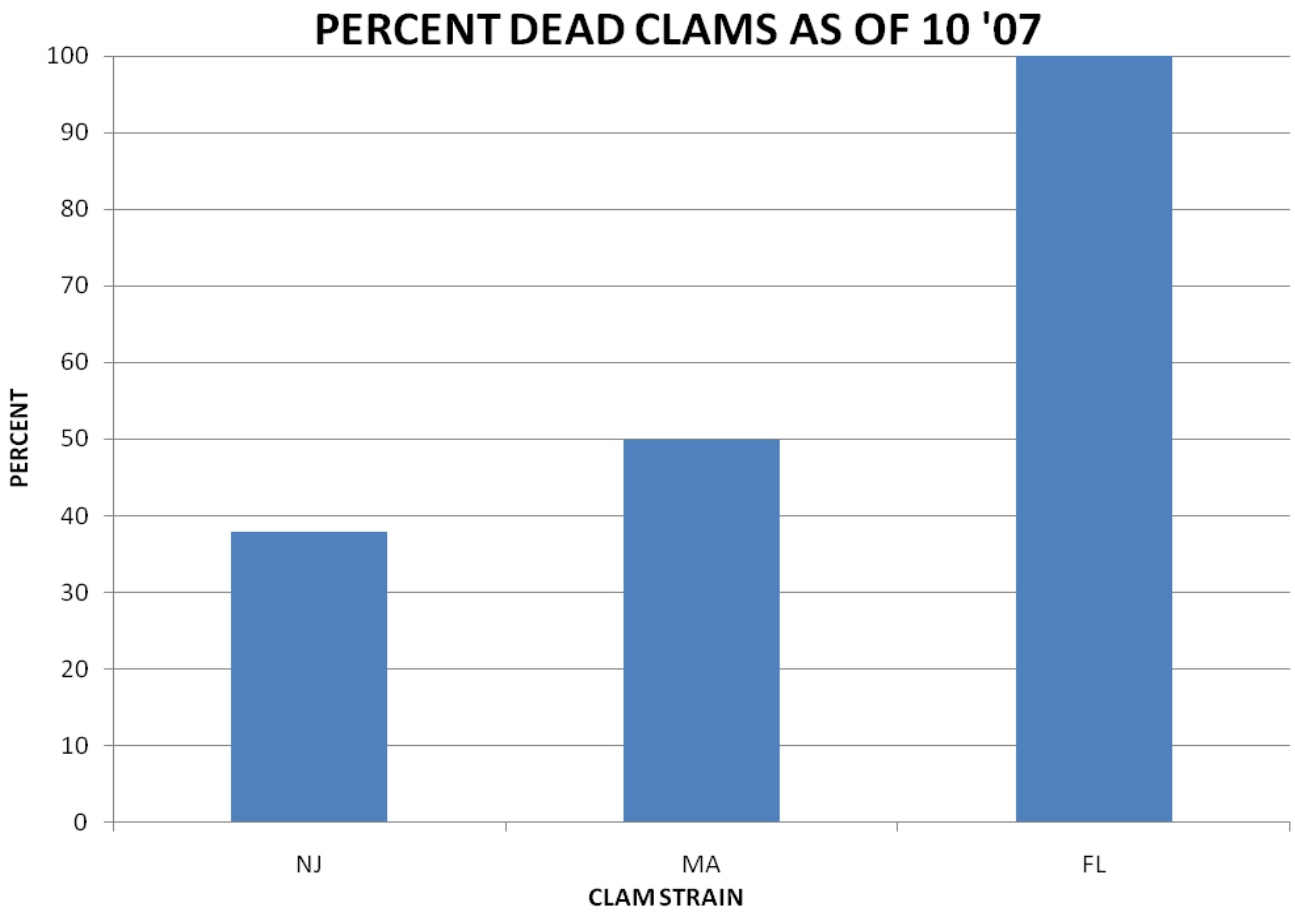
PERCENT DEAD CLAMS AS OF 10 '07
100
90
80
70
60
50
40
30
20
10
0
PERCENT
NJ
MA
FL
CLAM STRAIN

Figure 11.

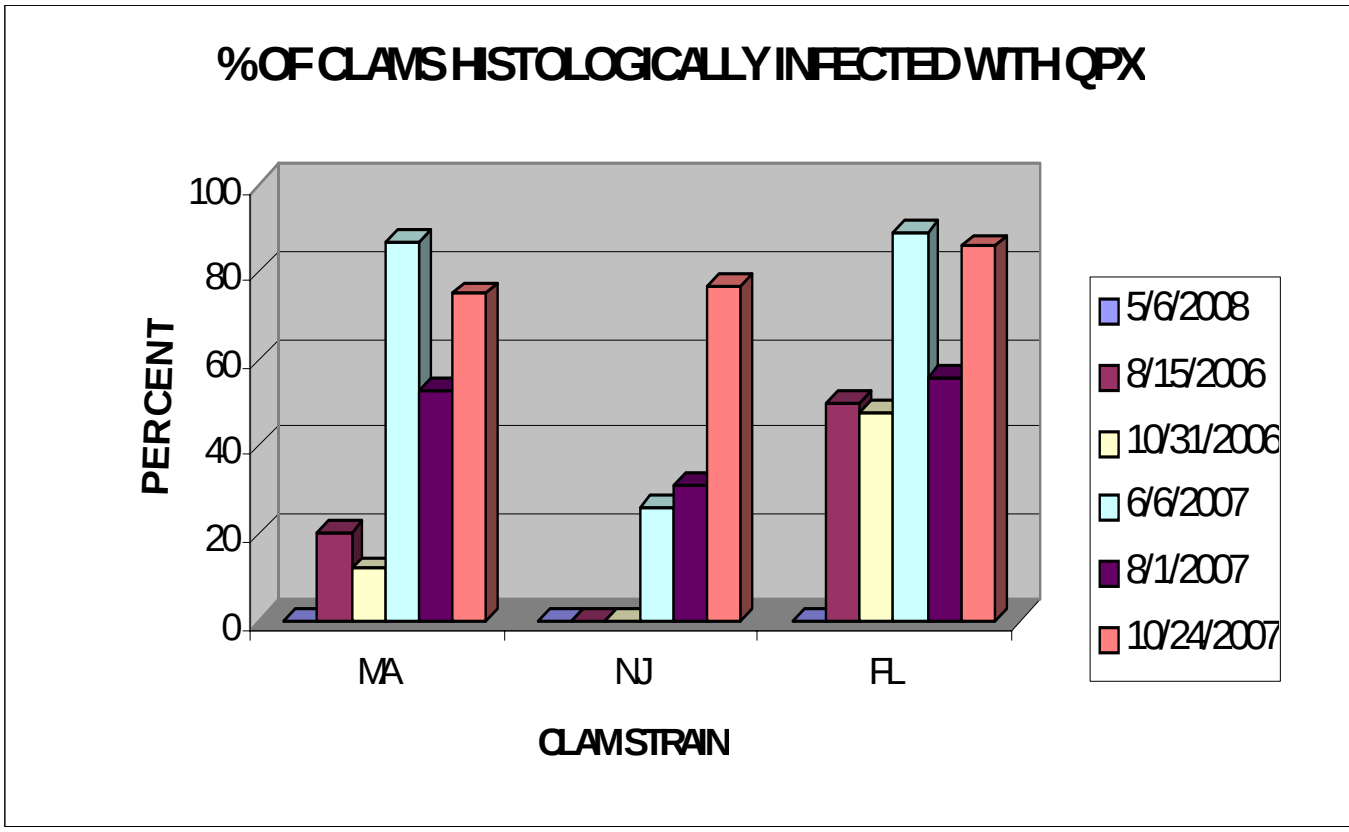
%OF CLAMS HISTOLOGICALLY INFECTED WITH QPX
PERCENT
100
80
60
40
20
0
MA
NJ
FL
CLAM STRAIN
5/6/2008
8/15/2006
10/31/2006
6/6/2007
8/1/2007
10/24/2007

Figure 12.

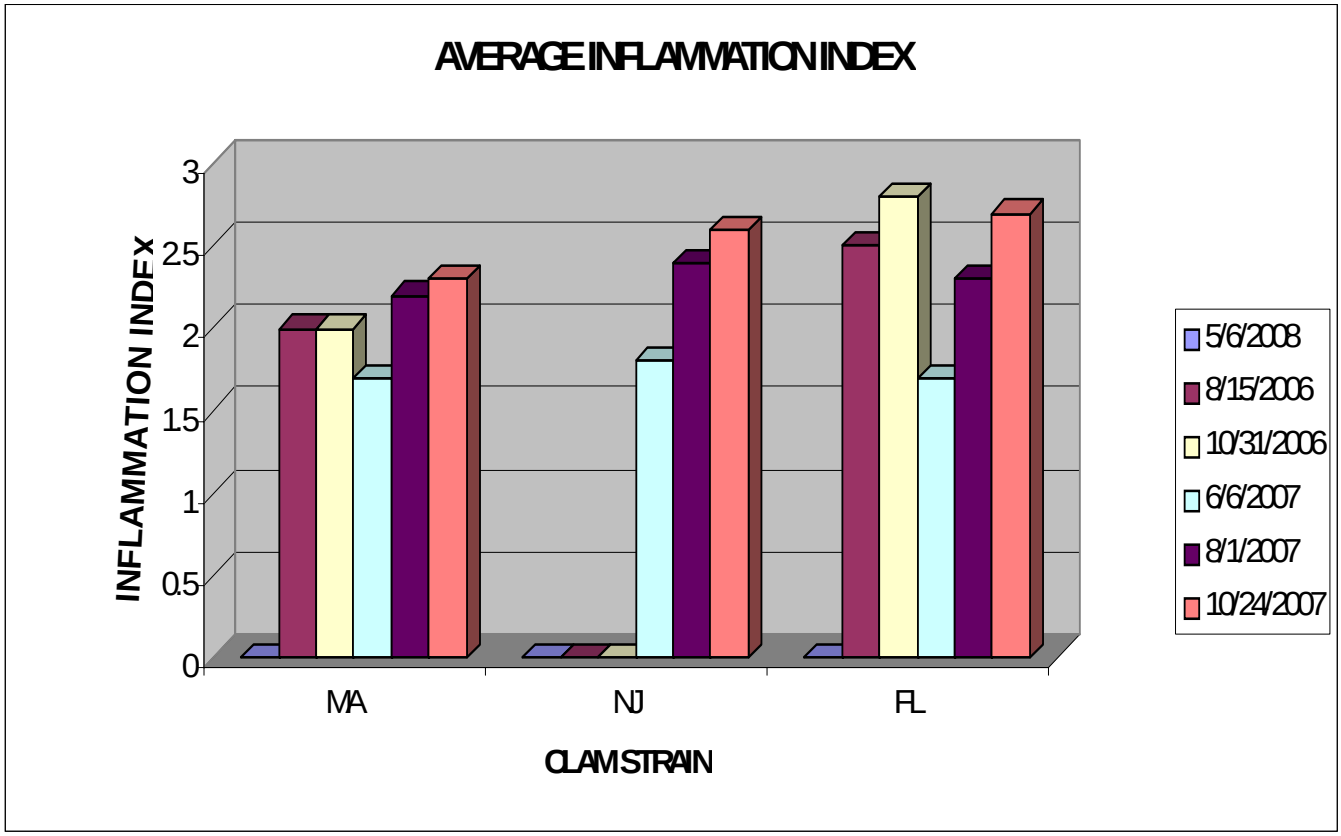
AVERAGE INFLAMMATION INDEX
INFLAMMATION INDEX
3
2.5
2
1.5
1
0.5
0
MA
NJ
FL
CLAM STRAIN
5/6/2008
8/15/2006
10/31/2006
6/6/2007
8/1/2007
10/24/2007

Figure 13.

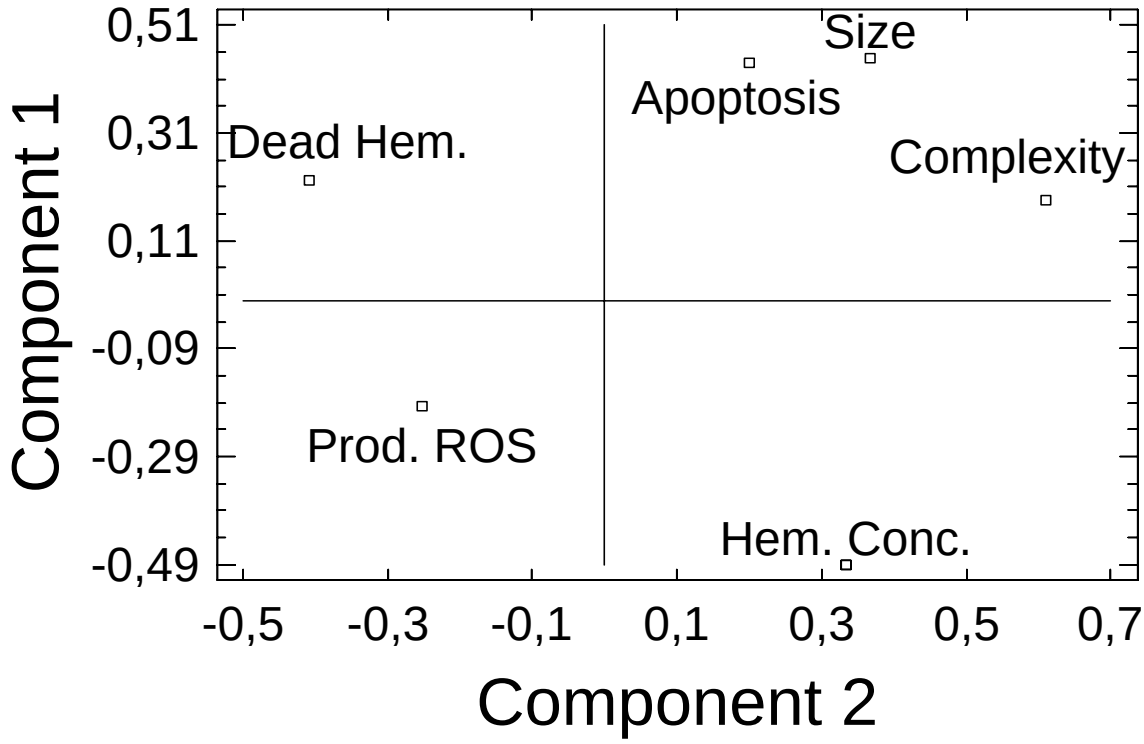
0,51
0,31
0,11
-0,09
-0,29
-0,49
Component 1
Size
Apoptosis
Dead Hem.
Complexity
Prod. ROS
Hem. Conc.
-0,5
-0,3
-0,1
0,1
0,3
0,5
0,7
Component 2

Figure 14.

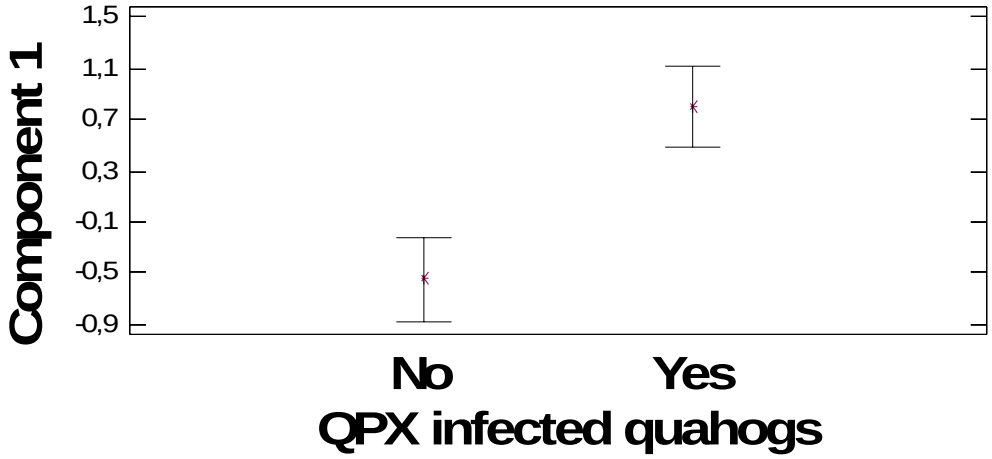

Figure 15.

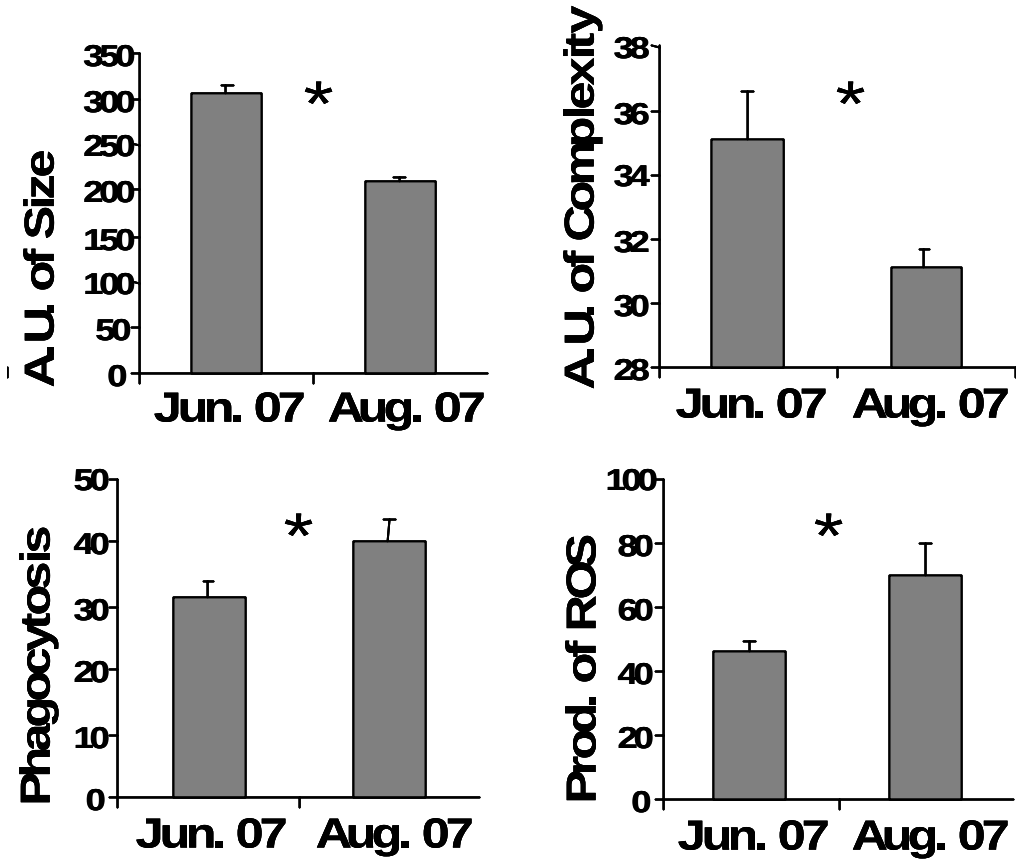
A.U. of Size
350
300
250
200
150
100
50
0
*
Jun. 07
Aug. 07
A.U. of Complexity
38
36
34
32
30
28
*
Jun. 07
Aug. 07
Phagocytosis
50
40
30
20
10
0
*
Jun. 07
Aug. 07
Prod. of ROS
100
80
60
40
20
0
*
Jun. 07
Aug. 07

Figure 16.

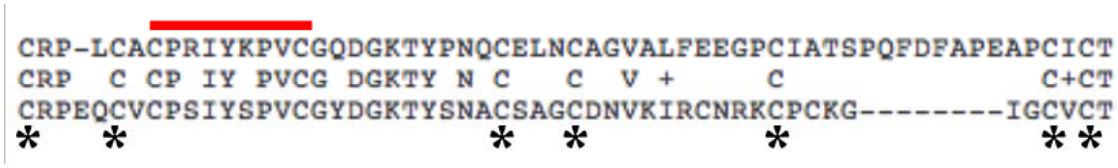
CRP-LCACPRIYKPVCGQDGKTYPNQCELNCAGVALFEEGPCIATSPQFDFAPEAPCICT
CRP C CP IY PVCG DGKTY N C C V + C C+CT
CRPEQCVCPSIYSPVCGYDGKTYSNACSAGCDNVKIRCNRKCPCKG--------IGCVCT
* * * * * * *

Figure 17.

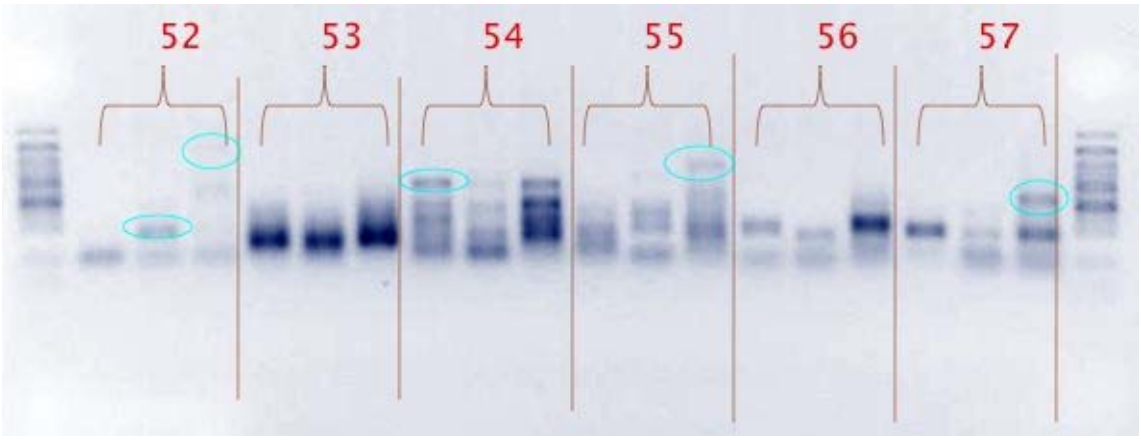

Figure 18.