## Title

Oyster Disease Research Program: Building Gene Expression-Based Predictors of Oyster Summer Mortality Syndrome



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Oyster Disease Research Program:
Building Gene Expression-Based Predictors of Oyster Summer Mortality Syndrome

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**Project Hypotheses**
Pacific oyster (*Crassostrea gigas*) fisheries on the U.S. West Coast, Mexico, France and Japan, are significantly impacted by high rates of oyster mortality in the summer months, in California ranging from 52-63%. This summer mortality syndrome (SMS) occurs predominantly in near-market ready oysters and seems to impact reproductive females the most. There is an urgent need for studies to investigate the metabolic and molecular basis of the syndrome, with the goal of identifying, and then producing summer mortality-resistant stocks. The specific hypotheses that we are testing is that changes in hemocyte gene expression could be used as a predictor of the onset of summer mortality, and that these changes in gene expression could be used as a predictor of the onset of the syndrome as well as providing insights into the pathophysiological basis of the disease.

**Project Goals and Objectives**
Research findings to date have made some advances in understanding the causes behind summer mortality syndrome (SMS), but we do not have the predictive tools needed to prevent mortality or to judge how genetic factors and the prevailing environmental conditions interact, and contribute to the susceptibility or resistance to summer mortality. Contemporary genomic technologies have the potential to address this complexity and also contribute to both the diagnostic and prognostic characterization of SMS in oysters in the field. Microarray-based gene expression profiling constitutes an open screen of genes, identifying those whose expression is regulated in relation to changes in the performance of the whole system. In this proposal, we will deploy a Crassostrea gigas microarray to generate new insights into the links between environmental change, gene expression, and the emergence of SMS. Specifically:

Aim 1 of the project will characterize the gene expression patterns exhibited by oysters during the early stages of SMS and will establish gene expression-based outcome predictors of SMS, that is sets of genes whose expression serves an indicator that the oyster is susceptible to SMS, thus alerting the grower to take remedial steps such as harvest or transfer the oysters to a less stressful location.

Aim 2 will investigate the association between genotype, gene expression, and the incidence of summer mortality.

Aim 3 will investigate the role of elevated temperature on the incidence of summer mortality.

Aim 4 will validate the gene expression-based predictors of the syndrome developed in aim 1 by examining this expression in oysters cultured at site in N. California.

Aim 5 will develop a quantitative RT-PCR method that could be used as a screening method to predict whether oysters at a particular field site were under the threat of a mortality episode.

**Briefly describe project methodology**

Field trials have shown that *C. gigas* inbred lines 51 and 35 exhibit significantly greater summer mortality than 51x35 hybrids grown at the same site. In this project, progeny from a 51x35 F2 family will be deployed in the field for growout. Since oysters are sessile, the same assemblage of animals can be sampled repeatedly over a period of months, thus allowing phenotype to be interpreted within the context of prior environmental events. Using an innovative repetitive sampling approach, we will interrogate the gene expression profile of individuals from this F2 family throughout the summer and will seek to link changes in gene expression with resistance or sensitivity to summer mortality of individuals. Repeated samples will be obtained from the animals using a non-sacrificial hemocyte sampling technique. Hemocyte RNA will be prepared, amplified, and then hybridized to a comprehensive *C. gigas* cDNA microarray. The expression data will be analyzed using a machine-learning algorithm, a data analysis technique developed for human cancer studies, which in a clinical setting, is used to identify gene expression patterns in tumor biopsies that correlate with the patient's prognosis. Exploiting this approach, we will classify the hemocyte RNA samples as belonging to oysters that survived or succumbed to mortality during the summer, and the algorithm will be asked to identify the gene expression patterns that correlate with, and are the most robust predictor of SMS. The gene expression changes in field animals will be interpreted within the context of laboratory simulated summer stress conditions imposed on oysters that exhibit differential sensitivity to SMS. We will also seek to extend the predictive capacity of gene expression screening approach to muscle tissue, which is more easily sampled by farmers in the field. In tandem will search for transcripts whose expression is significantly associated with the mortality QTLs identified in an associated ODRP proposal. Finally, we will establish a network of oyster growers in California to contact us at the first sign of SMS and then secure these animals for gene expression screening and these data will be used to validate and refine our predictive gene expression signature.

**Describe progress and accomplishments toward meeting goals and objectives**

Summer 2007 fieldwork

In summer 2007 we made 4 trips to Shelton, Washington at 2-3 week intervals from June 27 to August 28 to assess and sample the F2 family of oysters that were reared for summer mortality tracking. The first trip identified and sampled 205 oysters for repeated hemolymph sampling, and 137 oysters for possible tissue and histological sampling. All 342 oysters were bagged after each sampling and secured on the beach at Totten Inlet. During the following three sampling trips oyster bags were collected off of the beach at least 2 hours before peak low tide, and prepared for sampling either at the beach or taken up to Quilcene, WA for access to a lab with flowing seawater. Flowing seawater was necessary if the sampling involved redrilling and retagging the oyster's shell for hemolymph sampling; however, this was only necessary on the first two trips, a battery operated drill was used on the last two trips. Sampling on the beach was restricted to low tide hours due to the low tidal height of the experimental plot. To sample hemolymph, a small hole is drilled in the oyster's shell above the adductor muscle, and using a 25 gauge needle with 1ml syringe 100-200ul of hemolymph is removed from the oyster, and promptly placed in a microcentrifuge tube and frozen on dry ice. Samples were archived at -80°C thereafter. In total, we collected 768 hemolymph samples successfully from the 205 oysters that were selected for repeated sampling. 15 whole oysters were also sampled on each visit for additional analyses.

The incidence of summer mortality was exceptionally low in 2007 and only 31 out of the 342 monitored oysters died (9%) during the summer, a much lower number than the 50% that were expected to die according to historical data. In fact the summer of 2007 had very low rates of mortality across Washington State (Dan Cheney, Pacific Shellfish Institute pers. comm.). Statistical analysis of the mortality rates in animals that were sampled repeatedly for hemocytes versus animals that were not sampled for hemocytes indicated that there drilling and repeated drawing of hemolymph had no effect on mortality. This validates our drilling and repeated hemocyte sampling regime as a viable method for use in field-located oysters. At the same time we also assessed whether vertical position on the shore had an effect on mortality and statistical analysis confirmed that mortality is the same regardless of position on the shore.

Summer 2008 fieldwork

Oysters were sampled again in summer 2008 suing a similar methodology to that established in 2007. Cumulative mortality was greater in 2008 with 25% mortality within a cohort of 300 animals that we were monitoring. This was a slightly warmer summer and the maximum body temperature that we measured in field oysters was 37.5 C. This higher rate of mortality is important because we now have sufficient numbers of oysters that suffered mortality to undertake a more extensive gene expression screen.

Gene expression analysis

Summer 2007 dataset: The hemolymph samples from 7 oysters that did suffer mortality in summer 2007 were hybridized to the first iteration of *C. gigas* cDNA microarray. This cDNA microarray was constructed with the first set of sequenced cDNAs that were generated by a collaborative large-scale *C. gigas* EST sequencing project with the DOE Joint Genome Institute (JGI). From these sequences we selected 5,376 cDNAs for inclusion on our first iteration of the *C. gigas* microarray. The cDNAs were amplified by PCR and spotted onto glass slides at the microarray facility at USC. The array was validated by assessing the relative expression of different functional classes of genes across 12 oyster tissues. These data are valuable in the context of the current project as they provide insights into the functional roles of the different tissues and will help to guide interpretation of the metabolic role and response of different tissues in summer mortality syndrome. These data were presented as a poster at the National Shellfish Association Annual Meeting on April 6-10, 2008, by Maxine Chaney, the graduate student supported by a Sea Grant Traineeship on this project. This presentation won the Gordon Gunther award for best poster at the conference. We have been able to extract up to 1ug of total RNA from 200ul of hemolymph. Amplification of this RNA yields 30ug of amplified RNA which is sufficient RNA for hybridization to 15 microarrays. So RNA yield has not been a limiting factor in these experiments.

Despite the low mortality in 2007, we undertook a gene expression screen that compared the gene expression levels between 7 oysters that suffered mortality in 2007 and a corresponding number of oysters that survived the summer. Analysis of variance was used to test for statistical significance of expression differences between the two groups. A set of 60 genes exhibited significant gene expression differences between oysters that went on to survive or die that summer. Hierarchical cluster analysis supported this finding and showed a clear separation between the two groups in gene expression space. This finding was an important proof-of-principle because it confirmed that future differences in oyster survival were evident as patterns of gene expression in their hemocytes taken weeks before the animal died.

Summer 2008 dataset: The JGI completed sequencing of 150,000 *C. gigas* cDNAs from both their 5' and 3' ends in the summer of 2008. These sequences were complemented with nearly a million 454 reads from larval RNA samples. Clustering analysis of these sequences was used to generate a non-redundant set of >15,000 cDNA clones. From this set, 11,900 cDNAs were selected for inclusion on the current *C. gigas* microarray. The average length of these cDNAs is 2.5Kb and they were selected on the basis that they possessed a significant homology to genes that are in the public databases. These cDNAs will be re-sequenced by Illumina Genome Analyzer in January 2010, to provide a full-length cDNA resource. The 11.9K cDNA microarrays were printed in November 2009 and hybridization of RNAs from 30 mortality and 30 surviving oysters from summer 2008 are underway.

**Project Modifications**

The major challenge we have experienced is the unexpected low incidence of summer mortality in oysters in the summer of 2007. A consequence of this is that we obtained fewer RNA samples from animals affected by summer mortality that would compromise the detection of genes whose differential expression is linked to the syndrome. This forced us to sample again the same F2 family of oysters in summer 2008 so as to increase our sample size. This essentially put the project back 1 year and so we were unable to fulfill our goal of validating the gene expression data in other summer mortality field sites in California.

Given the unpredictability of mortality in the field animals we undertook a large-scale simulation of summer heat stress in animals in a controlled hatchery situation. A 51 x 35 F2 family of >400 2-year old oysters were brought into the hatchery in May 2008. The animals were separated into two conditioning holding systems and were provided with abundant algal food. The water temperature in each system was raised by 1 C every 2 days to simulate warming in the field. In one of the systems the oysters were kept submerged the entire duration of the experiment, while in the other system the animals were subjected to aerial emergence for 4 hrs a day. Aerial emergence was accomplished by using pumps on timers that would pump water out and into the holding tank at the same time each day. As water was pumped out of the tank, a large radiant heat panel positioned above the tank was switched on subjecting the animals to radiant heat, which raised their body temperature to ~28 C. Thus one set of animals were kept under constant submergence while the other set experienced the simulated ebb and flow of the tide and aerial emergence. Hemolymph samples were taken from every oyster at the beginning of the experiment and aliquoted for both genotype and expression analysis. Oysters were scored for mortality every morning and thus the time and temperature of death of each oyster was documented. The rate of mortality was significantly greater in the tidal system with 25% mortality observed at 30 days into the experiment (water temperature = 35 C), whereas just 12% of the submerged oysters were dead at the same time-point. Both groups of oysters experienced a precipitous rise in mortality when water temperature reached 36 C. The experiment was continued until every oyster was dead. Thus, we have a collected two large datasets that describe the thermal tolerance phenotype across an F2 family under two different heating regimes. These phenotypic data are being interpreted within the context of genotype (in collaboration with Hedgecock lab at USC) and gene expression by our laboratory.

We have begun extraction of RNA from the oysters that were sampled as part of the heating simulation study. Genotype data from these animals that is coming out of the Hedgecock lab indicates that there is a clear genetic basis for inter-individual differences in oyster thermal tolerance. Our gene expression data will complement this genetic data by providing paired expression and

genotype data for a subset of 100 oysters that are located at the tails of the distribution of thermal tolerance phenotype (ie. the 50 oysters exhibited the lowest thermal tolerance versus the 50 oysters that exhibited highest thermal tolerance).

In summer 2008, we subjected inbred lines of oysters to a variety of common environmental stresses that included, hypoxia, cold, heat, acidificaction, salinity, and oxidative stress. A database of gene expression responses to these controlled stresses will be generated and used to inform analysis of gene expression data generated from field oysters. Pattern matching techniques will be used to find similarities between field and lab expression data allowing gene expression patterns in field animals to be linked to specific environmental stresses, as tested in the laboratory.

**Publications**
Conference papers, proceedings, symposia
Title: Correspondence between tissue function and gene expression in *Crassostrea gigas*
Authors: Chaney, M. & Gracey, A.Y.
Date: 05/06/2008
Conference Title: National Shellfisheries Association Annual Meeting : A Century of Shellfish
Location: Providence, RI

Title: Exploring summer mortality disease characteristics in Crassostrea gigas through gene expression
Authors: Chaney, M. & Gracey, A.Y.
Date: 03/22/2009
Conference Title: 101st Meeting of the National Shellfisheries Association
Location: Savannah, GA

**Students**
Maxine Chaney
University of Southern California
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Degree program enrolled in: Ph.D.
Theses/dissertation title: Molecular Analysis of Oyster Summer Mortality Syndrome
Supported by Sea Grant funds? No response
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**Cooperating organizations**
None listed

**Awards**
Gordon Gunther best poster award at 100th Annual National Shellfisheries Association meeting in Rhode Island 2008.

**Keywords**
gene expression, oyster, summer mortality, heat