

Disease Resistance in the Eastern Oyster, *Crassostrea virginica*

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**Abstract:**

Marine shellfish are an “environmentally friendly agricultural commodity” (Scientific Aquaculture, 2006). Not only can they remove excess nutrients, which improves water quality, but they also represent a significantly growing economic market. This market, though, has recently seen reduced production in large part due to disease. The ability to find a resistant broodstock would be both environmentally and commercially important.

A two-fold approach was taken to examine the disease resistance in the Eastern oyster. First, the survival percentage of two broodstocks of Eastern oysters, Edgartown Great Pond (ETGP) and Tisbury, were compared. The ETGP oysters were historically exposed to a high disease level, while the Tisbury oysters had been exposed to minimal disease levels. Both groups were placed in a disease-filled environment, the Edgartown Great Pond, for a period of one year. During this period, two censuses were conducted to monitor survival rates. In addition, 60 oysters from each group were taken to sample hemolymph.

The second approach focused on the molecular aspect of the oyster’s immune system. In particular, the gene expression levels of cathepsin were monitored. Cathepsin is a lysosomal cysteine protease involved in immune functions such as antigen processing and proenzyme cleaving. cDNA was generated from total RNA using the reverse

transcription of extracted oyster hemolymph. The SYBR Green method of Quantitative Real Time PCR was used to determine the relative concentration of gene expression.

These values were normalized to the 18S gene using Real Time Data Miner Software.

The survival percentage of ETGP oysters was consistently greater than Tisbury oysters (99.26% versus 97.89% in June and 99.60% versus 99.27% in July). These rates correlate with molecular data that showed ETGP oysters possess 2.5 times the Cathepsin gene expression than Tisbury oysters. Both data sets suggest that the ETGP oysters could be important resources for broodstock given their improved survival.

### **1. Introduction:**

The Eastern Oyster, *Crassostrea virginica*, is a bivalve mollusk that is found along the Atlantic Coast of Mexico, Canada, and the United States. They are considered keystone species because of their abilities to form oyster reefs and improve water quality. A single reef can substantially increase the surface area by providing nooks and crevices that can become home to animals such as anemones, barnacles, and mussels. Oysters are excellent filter feeders that can filter up to 5 liters of water per hour. In addition to filtering plankton, oysters can remove excess nutrients, silt, and pollutants from the water. The removal of these harmful compounds from the water can improve the water quality and counteract eutrophication.

For these reasons, oysters are considered an “environmentally friendly agricultural commodity” that represented a 70 million dollar industry in 2001 (Scientific Aquaculture, 2006). However, that number has been declining every year since then. One of the causes of the fall off is a considerable decrease in oyster populations due in large part to disease. Some of these major diseases result from microscopic organisms

that inhabit the waters along the east coast of the United States. One of the major diseases is Multinucleated Sphere X (MSX), which is caused by a protozoan, *Haplosporidium nelsoni*. Another prevalent disease that has been affecting oysters is Juvenile Oyster Disease (JOD), which is caused by a group of *Rosebacter* proteobacteria called CVSP (Bivalve Disease, 2006). A third disease, Dermo, is caused by the protozoan parasite, *Perkinsus marinus*. Each of these diseases has their own symptoms, but they all ultimately end in death. With mortality rates between 40 and 90 percent, these diseases produce serious problems for the oyster industry (Infectious Diseases, 2004 and *Perkinsus marinus*, 2006).

One of the ways to combat these diseases is to identify what allows certain populations of oysters to survive in diseased water, while others do not. In particular, understanding the oyster's immune system is vital. This system is largely dependant on the ability to transport hemocytes through the oyster's open circulatory system. These cells are the mollusk equivalent of white blood cells and travel though a clear fluid called hemolymph. Oysters possess innate immune systems, which can only distinguish between "self" and "non-self" and will defend itself a in non-specific manner against all "non-self" particles. One of the non-specific defenses is phagocytosis by hemocytes. These cells recognize foreign particles, consume them, and degrade them using an oxidizing reagent, such as hydrogen peroxide (Combating Disease, 2003).

1	cagagacaga	gggacatact	agaaactagt	tagaagatga	attgtttgtg	gatagtgtgc
61	atgctagcgg	tggcgagggg	agcctctgtg	cagtctggaa	atgtccagtg	gtttgacctg
121	gaggcagcac	agaaacaccc	agaa <b>caacta</b>	<b>cacgtcctga</b>	<b>gggccaaggc</b>	<b>tgccatcaac</b>
181	<b>ttccagccct</b>	<b>acgaacaggc</b>	<b>ctggaaggag</b>	<b>ttcaagatcc</b>	tacacgacaa	aacttacaaa
241	<b>gctcttgagg</b>	aagaaatoca	cgcctttgag	at <del>ttt</del> caagg	aaaatgtaca	gaagattgag
301	gagcacaaca	aaatgtatca	cctcggcaag	aagtcctact	acatgggagt	taaccagttc
361	tccgatctga	aacacgagga	gtttgtgaaa	<b>tacaacggcc</b>	<b>tgaacaggac</b>	<b>atctctgaag</b>
421	<b>gacggaggat</b>	<b>gttcctcgta</b>	<b>cctggccgct</b>	<b>aaaaacctgg</b>	<b>ttgtccctga</b>	<b>ctcaatggac</b>
481	<b>tggaggacga</b>	<b>agggctatgt</b>	<b>caccccagtc</b>	<b>aaaaccagg</b>	<b>gtcaatgtgg</b>	<b>ttcctgctgg</b>
541	tcgtttagca	caactgggtc	tcttgaggga	caacactttc	gcaagtctgg	taaactgctt
601	tccctcagtg	agcagcaact	ggtggactgc	tccggatcat	tccgcaatga	aggttgtaat
661	ggaggactta	tgataatgc	attcaaatat	atcaagtctg	taggaggcct	tgagactgaa
721	gaggattatc	catacaaggc	caagcaaaat	gactgtaatt	ttgatgactc	taaagttgct
781	gctaccga					

Figure 1. The sequence above is the 788-nucleotide sequence for a cathepsin-like protein in *Crassostrea virginica* (Acc# CD648991). The location of the CathL 5' Forward primer is marked in blue text and the location of the CathL 5' Reverse Primer is marked in red text. The highlighted yellow text is the original EST that was BLASTed against the NCBI database.

A molecular approach was taken to examine the oysters' immune system. This was achieved by focusing on gene sequences that code for proteins that are involved in oyster immune response. A National Center for Biotechnology Information (NCBI) "blastn" search of a *C. virginica* expressed sequence tags (EST) database produced several of these sequences. One EST had a high homology to a 788 base pair sequence that coded for a protein known as cathepsin (Acc# CD648991). Cathepsin is a type of lysosomal cysteine protease that is involved in a few immune functions (Turk 2001). One type of immune function is antigen processing, in which cathepsin will degrade foreign proteins that have invaded the cell. The leftover polypeptide fragments are then used as antigens, which are placed on the outer side of the cell membrane. These antigens signal the other cells to phagocytize the infected cell. Another immune connection is proenzyme cleaving. This process involves a cathepsin cutting a region on a proenzymes that results in the activation of that enzyme. Possible enzymes that could be involved in this pathway are antimicrobial enzymes and enzymes that are involved in oxidation.

## **2. Materials and Methods:**

### *2.1 Oysters:*

Adult Eastern oysters, *Crassostrea virginica*, were removed from two locations on Martha's Vineyard, Massachusetts. One group was taken from the Edgartown Great Pond (ETGP), which has historically contained oyster diseases. The second group was taken from the waters off the coast of Tisbury, Massachusetts. This area has had minimal levels of oyster diseases. Both groups of oysters were forced to reproduce and the offspring were collected.

Each group of offspring was divided into eight mesh bags so that there were 250 - 300 oysters per bag. On July 26, 2005, these were placed along two mooring lines in the Edgartown Great Pond. Two censuses were conducted to monitor mortalities, one in June 2006 and one in July 2006. In addition, 60 oysters were removed, thirty during each census, to have hemolymph sampled.

### *2.2 RNA Extraction:*

RNA was extracted from the hemolymph tissues using a Tri Reagent kit from Molecular Research Center Inc. The protocol that accompanied the kit was followed to extract the RNA. The isolated total RNA was suspended in 20  $\mu$ l of DNase and RNase free water and stored at  $-80^{\circ}\text{C}$ .

### *2.3 Reverse Transcription:*

Reverse transcription was used to create a stable cDNA sequence from the fragile RNA sequence. In order to do this a 15  $\mu$ l reverse transcription master mix was created. This mix included 4  $\mu$ l of AMV 5x Buffer, 8  $\mu$ l of 2.5 mM dNTPs, 1  $\mu$ l of AMV Reverse

Transcriptase, 1 µl of Oligo dT Primer, and 1 µl of H<sub>2</sub>O. Once the mix was created, 5 µl of the total RNA extract was instant-incubated in the thermocycler (MJ Research PTC-200 Peltier Thermocycler) at 75 °C for 5 minutes. Then the RNA extract was placed on ice for 5 minutes before being added to the master mix. Finally, the combined solution was vortexed, spot spun, and incubated at room temperature (22 °C) for 10 minutes. Next, the tube was instant-incubated at 37 °C for 1 hour followed by 95 °C for 3 minutes. The final cDNA product was stored at -20 °C.

#### *2.4 Primer Design:*

Primers were designed using Integrated DNA Technologies' Primer Quest. This program used the known cathepsin nucleotide sequence to design primer sequences. The forward primer, CathL\_fw (5'- ATG TCC TGT TCA GGC CGT TGT A - 3') had an annealing temperature of 60.1 °C and the reverse primer, CathL\_rv (5' - ACA GGC CTG GAA GGA GTT CAA GAT - 3') had an annealing temperature of 58.6 °C. Together, these primers produced a polymerase chain reaction product that was 218 nucleotides. These primers and product sequences can be seen in Figure 1.

#### *2.4 Polymerase Chain Reaction:*

Polymerase Chain Reactions (PCR) were conducted on the stored cDNA that was generated from the hemolymph. These reactions were used to amplify the cathepsin sequence so as to be visible when run in an agarose gel. A 25 µl (per reaction) PCR master mix was created by combining 16.5 µl of H<sub>2</sub>O, 5 µl of GoTaq 5x Buffer, 2 µl of 2.5 mM dNTPs, 0.25 µl of the Forward Primer (CathL\_fw), 0.25 µl of the Reverse Primer (CathL\_rv), 1 µl of GoTaq Polymerase. Next, 24 µl of the master mix were added

to a PCR-capable micro-centrifuge tube or plate. Finally, 1  $\mu$ l of the oyster hemocyte cDNA template was added to the tube before it was placed in the thermocycler. The PCR cycles were as follows: 1) 95  $^{\circ}$ C, 5 min; 2) 95  $^{\circ}$ C, 1 min; 3) 60  $^{\circ}$ C, 1 min; 4) 72  $^{\circ}$ C, 2 min; 5) 72  $^{\circ}$ C, 10 min (repeat steps 2-4, 40 times). This PCR reaction took between 3 and 4 hours and once finished, the PCR products were stored at -20  $^{\circ}$ C.

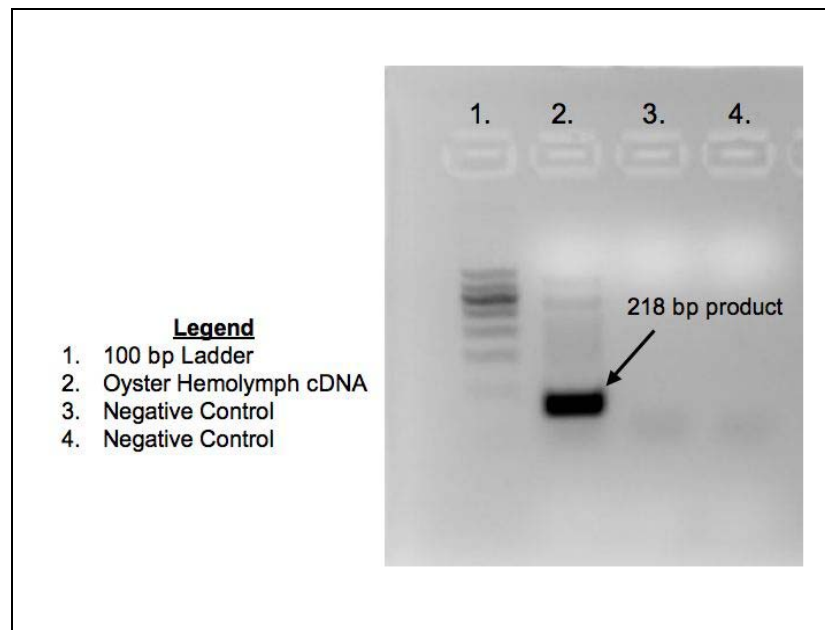


Figure 2. The image above is an example of a 1% Agarose gel that was run to determine the presence of the cathepsin sequence in an oyster hemolymph cDNA sample. Lane 1 contained the 100 bp ladder, lane 2 contained the oyster hemolymph cDNA sample, and lane 3 and 4 contained negative control samples. At least two negative control samples were run with each PCR reaction. The negative controls only lacked a cDNA template. The oyster hemolymph cDNA sample contained the cathepsin sequence due to the presence of a 218-nucleotide sequence.

### 2.5 Gel Electrophoresis:

Gel electrophoresis was the next step, which was used to determine the presence of the cathepsin sequence in the cDNA sample. A 1% (1.33% m/v) agarose gel was created by adding 1 g. of agarose to 75 ml of 1x TAE. This solution was heated for 1.5 minutes in a standard microwave. Then it was removed, shaken, and placed back in the microwave to be heated for another 1.5 minutes. After allowing the solution to cool, 5.5

$\mu\text{l}$  of Ethidium Bromide was directly added to it. Next, the solution was poured into a mold and allowed to polymerize.

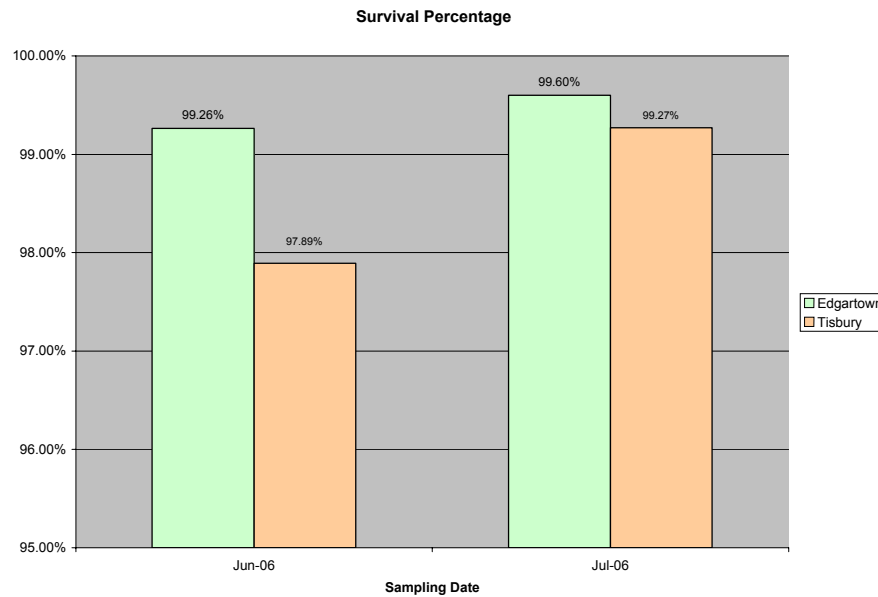
Once the gel had solidified, 25  $\mu\text{l}$  of the PCR product were loaded into each well. A ladder was also added in the first well. The gel was run for approximately 20 minutes at 100 volts. Afterward, the cDNA bands were visualized by exposing them to ultraviolet light using the TL-2000 Ultraviolet Translinker. Once the bands were spaced out enough to distinguish between them, the gel was placed on top of the light box within the Alpha Innotech Corporation MultiImage Light Cabinet. Using the light cabinet, a picture of the gel was taken and used to determine the size of the bands. Figure 2. shows a picture of one of the gels. The correct size band for the cathepsin sequence in oyster hemocytes is shown in lane 2.

#### *2.6 Quantitative Real Time Polymerase Chain Reaction:*

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) was conducted using the SYBR green method (Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step, Stratagene). A 25 $\mu\text{l}$  (per reaction) QT-PCR master mix was created by combining 10.3  $\mu\text{l}$  of H<sub>2</sub>O, 12.5  $\mu\text{l}$  of 2x SYBR Green Mix, 0.1  $\mu\text{l}$  of the Forward Primer (CathL\_fw), and 0.1  $\mu\text{l}$  of the Reverse Primer (CathL\_rv). Next, 24  $\mu\text{l}$  of the master mix were added to a QRT-PCR 96 well plate. Finally, 1  $\mu\text{l}$  of the 1:5 diluted oyster hemocyte cDNA templates were added to each well before it was placed in the thermocycler (MJ Research DNA Engine 2 Opticon Continuous Fluorescence Detector). An annealing temperature of 55 °C was used.

### 3. Results:

#### 3.1 Census Results:



Graph 1. This graph illustrates the average survival percentage of each group for both censuses.

Censuses of the oysters that were placed in the Edgartown Great Pond were conducted in June 2006 and July 2006. Each census consisted of counting the total number of live and dead oysters per bag. These values were recorded and totaled for both groups. Table 1 and Table 2 show the raw data from both censuses for each group. These tables also show the survival percentages, which were calculated by dividing the number of oysters that were alive by the total amount of oysters that were in the bag. The average survival percentage for each group is shown in Graph 1. This graph breaks down the survival percentage into groups of oyster populations and census dates. Though not statistically significant, the survival percentage of ETGP oysters were consistently greater than Tisbury oysters, 99.26% versus 97.89% in June and 99.60% versus 99.27% in July.

Edgartown Great Pond Oysters								
Bag	Initial Deployment Number	Alive	Dead	Percent Alive	June Redeployment Number	Alive	Dead	Percent Alive
19	177	178	0	100.00%	298	295	1	99.66%
15	177	175	0	100.00%	298	294	0	100.00%
29	177	179	0	100.00%	298	297	0	100.00%
61	177	176	0	100.00%	298	294	1	99.66%
28	356	374	0	100.00%	298	297	0	100.00%
49	356	350	0	100.00%				
59	356	352	0	100.00%	295	292	0	100.00%
5	319	291	27	91.51%	291	280	11	96.22%
25	316	318	0	100.00%	318	313	0	100.00%
7	335	312	0	100.00%	312	313	0	100.00%
55	321	312	0	100.00%	312	308	0	100.00%
35	299	296	1	99.66%	296	287	0	100.00%
57	318	310	0	100.00%	310	306	1	99.67%
<b>Average</b>	<b>283.38</b>	<b>278.69</b>	<b>2.15</b>	<b>99.32%</b>	<b>302.00</b>	<b>298.00</b>	<b>1.17</b>	<b>99.60%</b>
<b>Total</b>	<b>3684</b>	<b>3623</b>	<b>28</b>		<b>3624</b>	<b>3576</b>	<b>14</b>	

Table 1. The census data for each bag that contained Edgartown Great Pond oysters.

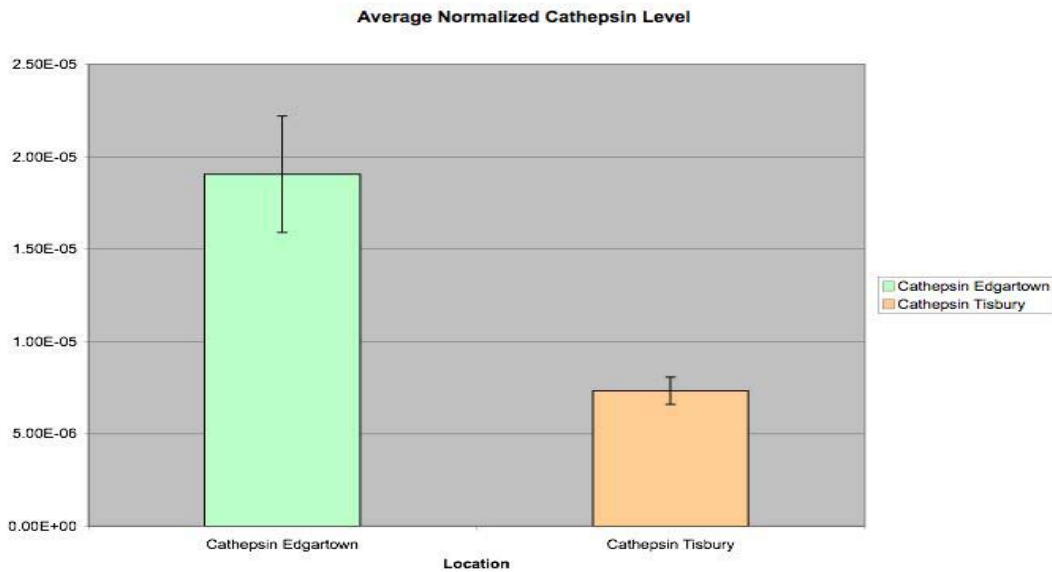
Tisbury Oysters								
Bag	Initial Deployment Number	Alive	Dead	June Percent Alive	June Redeployment Number	Alive	Dead	July Percent Alive
60	249	229	1	99.57%	229	231	0	100.00%
62	251	238	0	100.00%	233	235	0	100.00%
30	251	252	0	100.00%	252	253	1	99.61%
20	251	255	0	100.00%	255	228	0	100.00%
22	251	244	0	100.00%	244	249	1	99.60%
32	274	216	60	78.26%	216	209	9	95.87%
12	218	217	2	99.09%	217	215	1	99.54%
46	272	262	1	99.62%	262	272	0	100.00%
58	283	284	4	98.61%	284	279	3	98.94%
4	281	267	1	99.63%	267	277	2	99.28%
56	285	281	0	100.00%	281	274	4	98.56%
<b>Average</b>	<b>260.55</b>	<b>249.55</b>	<b>6.27</b>	<b>97.71%</b>	<b>249.09</b>	<b>247.45</b>	<b>1.91</b>	<b>99.22%</b>
<b>Total</b>	<b>2866</b>	<b>2745</b>	<b>69</b>		<b>2740</b>	<b>2722</b>	<b>21</b>	

Table 2. The census data for each bag that contained Tisbury oysters.

Sample Number	1	2	3	4	5	6	7	8	Average
Edgartown	1.82E-05	3.39E-05	1.88E-05	1.33E-05	5.45E-06	1.27E-05	2.34E-05	2.67E-05	1.905E-05
Tisbury	8.47E-06	8.08E-06	5.47E-06	5.96E-06	6.90E-06	7.01E-06	5.10E-06	1.16E-05	7.32E-06

Table 3. This table contains the normalized data that was outputted from the Real Time Data Miner. The data values possess no unit of measurement and only used for comparisons.

### 3.2 Quantitative Real Time Polymerase Chain Reactions Results:



Graph 2. This graph shows the average normalized cathepsin values for Edgartown Great Pond oysters and Tisbury oysters. The error bars represent the average standard error for each group.

Quantitative Real Time PCR outputted the raw concentrations of the cathepsin sequence for each hemolymph sample. These values were placed into a software program, called Real Time Data Miner, which normalized the data to the 18S gene. The outputted normalized values for each of the eight hemolymph samples are listed in Table 3. The averages, which were  $1.91 \times 10^{-5}$  for ETGP oysters and  $7.32 \times 10^{-6}$  for Tisbury oysters, are also listed in Table 3 and shown in Graph 2. The ETGP oysters had a 2.5 times greater expression level of the cathepsin gene than the Tisbury oysters, which was statistically significant (Students two sample, equal variance t-test, two tail,  $n=8$ ,  $p=0.002764$ ).

#### **4. Discussion:**

The ETGP oysters were historically exposed to a high level of diseases, while the Tisbury oysters came from an environment that had minimal disease levels. The diseases that the ETGP oysters were exposed to had high mortality rates, which led to selective reproduction due to the fact that only those oysters that survived could reproduce. This historical difference in exposure levels led to select populations of oysters that possessed a difference in their response to the various diseases.

The census results were consistent with this hypothesis. Over 6,000 oysters were sampled in each census and the bags with ETGP oysters had greater survival percentages than the bags with the Tisbury oysters. There are two inconsistent data points that need to be mentioned. Bag 5, which was composed of ETGP oysters, had 27 dead oysters in the June 2006 census and bag 32, which was composed of Tisbury oysters, had 60 dead oysters in the June 2006 census. These points are considered extreme outliers since only 1 and 9 other oysters, respectively, died from the ETGP and Tisbury groups, respectively, in that census and these totals represented 96% and 87% of the dead oysters total from each group for that census.

There are two possibilities that could have produced these results. Both bags 5 and 32 were next to each other and were the last two bags on the rope line. These bags may have not sunk properly, which would have resulted in the oysters being exposed to high temperatures on the surface of the water. Another possibility is that these two bags were beached on the shore of the Edgartown Great Pond. If either possibility occurred, it would have happened for only a brief time since neither bag had a complete die-off of oysters.

The quantitative real time PCR results also agree with the difference in oyster immune capacity hypothesis. The raw fluorescence data was inputted into the Real Time Data Miner to be normalized to the 18S gene, which is constitutively expressed in oyster hemocytes. This eliminates the need to run serial dilutions of a RNA sample. Instead, the Real Time Data Miner uses efficiencies and  $C_T$  values to determine the gene expression values (Sheng 2005). The normalized values were achieved by dividing the cathepsin value by the 18S value for each sample. The final outputted values are unit-less and only used for comparisons, not as stand alone values.

The average ETGP normalized cathepsin level was greater than the Tisbury normalized cathepsin level, which agrees with the hypothesis that there is a difference in immune function between these groups of oysters. In this case, the difference is the expression level of cathepsin. The elevated cathepsin expression levels in ETGP hemocytes suggest that there are more activated enzymes and antigens processed.

A continuation of both approaches to this experiment would be the next step. Censuses would continue to be conducted on the oysters that were placed in the Edgartown Great Pond. Continuing to monitor the survival of the oysters through August and September, which are the warmer water months, would provide a better reflection of the oyster' immune capacity because the major diseases have a higher prevalence in warmer waters.

Enlarging the sample size is one of the next steps for the molecular approach. Only eight hemocytes samples were used from each group. There were over 6,000 oysters that were placed in the field and each could provide a hemolymph sample. However, collecting hemolymph from a sample size that large would be impractical. A

sample size of 96 is not only practical for sampling purposes, but would also fill the QRT-PCR plate. Also different genes, other than cathepsin, that possess immune connections could be studied. One possible gene that could be studied in the same manner of cathepsin could be the High Mobility Group gene, which is known to mediate responses to infection.

In conclusion, both sets of data suggest that oysters that have survived stressors (i.e. disease pressure) could be an important resource for broodstock, given their improved survival rate. In addition, the gene expression data could indicate that naturally selected for oysters (ETGP) possess a greater immune capacity. However, more research is needed to better understand the direct relationship between gene expressions and the immune functions of *C. virginica*.

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