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Differential Gene Expression between Wild and Restoration Populations of the Eastern Oyster

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DIFFERENTIAL GENE EXPRESSION BETWEEN WILD AND RESTORATION

POPULATIONS OF THE EASTERN OYSTER**.**

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Abstract

Crassostrea virginica, the Eastern oyster, is a marine bivalve that filters plankton and other particles from the water it inhabits, and thus it is important for maintaining water quality. The oyster population has been declining in the past decades on the east coast, including around New York. The decline was initially caused by overharvesting but over time pollution and introduced diseases in the marine environment have become major stressors on the oysters' survival in the wild. The purpose of the study is to use genetic techniques to inform oyster restoration programs about how different oyster strains are responding to environmental conditions. I am comparing Maine hatchery oysters' to wild oysters' genetic responses, by examining their RNA, which represents the DNA that is functional. My goal is to isolate RNA from the oyster and use reverse transcription to convert RNA to cDNA. This cDNA is used in the real time quantitative polymerase chain reaction to allow me to quantify the gene expression data and observe differences among oysters from different sources grown in different areas, including Governors Island, Bush Terminal, and Soundview Park. Future work will examine both wild and hatchery-raised oysters from each site, using a paired sampling design, to directly compare the responses between these two groups of oysters. This design will control for differences in environmental conditions among sampling sites.

Introduction:

The Eastern oyster, *Crassostrea virginica*, is a bivalve classified in the phylum of Mollusca and family Ostreidae. It contains a central adductor muscle, which has a purple pigmentation that differentiates this oysters from others (NOAA Fisheries Eastern Oyster Biological Review Team,2007). The eastern oyster is native from the Gulf of Mexico and part of the eastern coast of North America. They have asymmetrical rough shells, which can change in shape and thickness depending on the environment (NOAA Fisheries Eastern Oyster Biological Review Team,2007). It can grow up to about eight inches long. Furthermore, *C. virginca* is a protandric organism, meaning that it has sequential hermaphroditism, changing sex at some point in their life stage. They have a lifetime average of 20 years old (Insite Horizons, LLC.). It is known that their reproductive cycle begins with the spawning of the gametes into the water column, later the free-swimming larvae are formed, which developed a "foot" required to seek for a hard substrate, which is essential for their maturation and where metamorphosis occurs. Moreover, the reproductive cycle depends on a combination of physiological factors of the environment such as temperature and salinity. This organisms tend to habitat shallow bays, estuaries and salty waters that range from 8 to 35 feet in depths, optimum temperatures range from 20 to 30º C (Stanley and Sellers 1986) and salinity about 14 to 28 psu; and are mainly found in intertidal zones and nearshore estuaries.

They are considered keystone species because they are essential for maintaining the structure of the ecological community they live in, as well as the fact of providing key ecological services to the marine ecosystem. Moreover, they mainly

feed on phytoplankton and algae, reducing its biomass (Langdon and Newell 1996),

preventing hypoxia. At the same time, we can refer to them as engineering species

since they modify the physical environment where they live. In the first place, they

create their own niche, forming what is known as oyster reefs, which is composed of

an accumulation of shells substrate that serves as a suitable habitat for other aquatic

organisms (NOAA Fisheries Eastern Oyster Biological Review Team,2007). In

addition, it has been discovered that oyster beds serve as essential coastal barriers,

reducing erosion levels in shorelines. Nowadays there is a notorious increase in

flood vulnerability related to the rise in sea-level, increase in numbers of storms and

erosion of the coast (Christine et al. 2016). This issue is believed to be related to the

reduction of coastal barriers that are created by living organisms such as coral reefs

and oyster reefs. In one of the previous studies on *C. virginica*, researchers analyzed

whether the American oysters beds indeed could function as a real potential form

to protect from floods by decreasing wave energy, and test the relation between

increasing wash of sediments in coastal ponds and over-harvesting of the American

oyster (Christine et al.2016). In order to do so, they performed reconstructions of

storms action in three ponds located in the outer bay of the Harbor of New York

City: Seguine Pond, Arbustus Lake, and Wolfes Pond. The observations made gives

insight that oysters reef serve to break waves when waves pass over the reef.

Furthermore, it was also concluded that there is a high correlation between

increasing metals levels and land clearance due to human activities, by using

methods such as dredges channels placed in the harbor, which could also have had

an impact at the sites. Modeling results showed that the presence of oyster reefs

reduced the waves' ability to carry and transport coarse-grained sediments. In conclusion, the recent destruction of oyster beds is found to be a potential explanation of high flood in the present.

    Nowadays the eastern oyster population has been declining in NYC and other parts of America since early 1900, being today 0.01% what they once were (NYC Oyster Monitoring Report). As mentioned above the growth of the organism highly depends on the environmental condition mentioned before, as well on the food supply available. The eastern oysters are filter feeders and their primary food is phytoplankton and suspended detritus matter (Langdon and Newell 1996). The filter-feeding rates can be adjusted depending on the size of the organism and the food available. *C. virginica* has reported having filtration rates ranging from 1.5-10.0 L h-1g-1 dry tissue weight (Stanley and Sellers 1986; Newell and Langdon 1996). Since oysters are filter feeders they are essential for enhancing water quality, reducing its turbidity, and offer food to other organisms. They feed by pumping water through their gills and in the process the trapped particles of food, sediments, chemical and contaminants ("Oysters | Chesapeake Bay Program"). However, there are several factors that contribute to this oyster population decline; one of the most important is due to collecting by humans. One remarkable event that led to the dramatic decline, near to extinction, of the *C. virginica* population, was the European colonization with the exacerbation of activities such as over-harvesting and fishing (Rothschild et al.1994). As a human, we depend on some services from the marine ecosystem and often bear the consequence of great impacts on them in a direct or indirect way. The decline of *C. virginica* population is also related to the

reduced water quality, increase of disease pressure, overfishing, excessive

harvesting and habitat destruction (Rothschild et al. 1994). Water quality has been

deteriorated due to increased pollution. We can also observe that ocean acidification

has been elevating as a result of rising atmospheric CO_2 (Boulais 2017).The change

in pH of the water to higher OH percentage has been proven to have an impact on

the oyster reproduction, which may result in negative effects on the organisms that

rely on these keystone species(Boulais et al. 2017).

         On the other hand, eastern oysters are facing growing disease pressure from

protozoan parasites Minchinia Nelson " MSX" and Perkinsus marinus "Dermo",

which was passed without noticed until 1960 in the Chesapeake Bay (Rothschild B.J

et al. 1994). The outbreak of these parasites is related to the increased fishing

practices of the oyster such as the Hand tongs and dredges (methods of harvesting

oysters), which damaged the physical integrity of the oyster. Most parts of the reefs

have suffered from being scraped . Therefore, oysters bed are now found to be flat

and mainly composed of a thin layer of shells of dead and live organisms, which in

turn result in a decrease in surface area and leads to the formation of a more

hostile habitat (retrieved from Chesapeake Bay site), affecting the organisms that

rely on them for shelter. In the Hudson River estuary, health conditions have been

improving in the last few years. However, there are still high levels of bacteria and

of sewer outflow, affecting the survival of this organism (retrieved from Hudson

River Park site). The appearance of these parasites resulted in high rates of

mortality and slower growth rates; it is believed that the transmission of these

diseases is influenced by the changes in temperature and salinity. Moreover,

previous studies have shown that there is about 50% decline in oyster growth, that their quality has also been affected and in equally form the habitat and substrate were they grow (Rothschild B.J et al. 1994). Furthermore, other of the factors influencing eastern oyster population is the intensive land use, replacing forestland increasing the number of sediments and nutrients that enter the rivers and streams, which degrades water quality and create harsh conditions for the aquatic organisms (Miller et al.2002). On one hand, the algae feed on those nutrients producing algae blooms creating low oxygen conditions inducing dead zones (Glibert et al.2007), affecting the growth and development of the *C. virginica*. As a whole these factors combined increase the level of stress of the organism, leading to higher susceptibility to diseases.

Various attempts of restoration have been having low rates of success; oysters are not being reestablished to the levels that existed during the 1800s. Researchers had been trying to study and analyze the factors that produce such a decline. The Chesapeake Bay and the Hudson River estuary are places where the decline has been noticed more significantly (Torben c. ET AL. 2016)(Eastern Oyster Biological Review Team 2007). Considerable restoration efforts in NYC are being led by the Billion Oyster Project and the Nature Conservancy. They have monitored seven oyster restoration sites throughout New York Harbor and measured oyster performance in terms of their growth, survival, reproduction rate and other factors that affect their quality of life such as. Moreover, they observed the abundance and biodiversity of other species to understand the effect of eastern oysters in other species population such as disease, predators, water quality, phytoplankton. They

attempt to do reef construction and monitor them. For measuring oyster survival

they used tagging techniques.   Oysters used in cages to perform reef restoration are

from hatcheries, meaning that the hatching of the eggs was facilitated under

artificial conditions.

This leads us to question why and what we can do about it. How do these

oysters from hatcheries differ in terms of their genetics and evolutionary response

to their local environment compared to the wild type oysters, and moreover how

does their gene expression differs in relation to wild oysters? In our research, we

want to study the influence of environmental factors in the activation and silencing

of certain genes in oysters under different stressful conditions, and compare wild

oysters' responses to those used in restoration programs. We study changes in gene

expression patterns, which are influenced by the environment but can also be

heritable, and do not involve changes in the DNA sequence. If stress responses of

wild and hatchery oysters differ greatly, this result would suggest that the hatchery

oysters vary greatly from the wild oysters in their ability to handle stress, which

might impact their ability to survive and grow in the natural environment. This

result would not be surprising, given that the hatchery environment is expected to

lead to artificial selection of genotypes that may be ill-suited to the wild. Early life

experiences can also lead to fixed patterns of gene expression, so I won't be able to

disentangle the heritable from the plastic effects of any differential gene expression

between hatchery and wild oysters.

We have already sampled oysters from three different environments in New

York City, including wild oysters and oysters from the Billion Oyster Project

restoration cages. The restoration oysters are from a hatchery in Maine, where the environment is very different from that in NYC. The question is when raised under similar environmental conditions, do the hatchery oysters respond differently than do the wild oysters? And are there locations where the wild oysters and caged oysters share the same stress response? Ideally, we want to be able to choose hatchery stocks that respond in a similar way to the environment as the wild oysters. That way, we can best restore the population so that the individuals in the population are similar to the wild, natural stock.

To address these questions, I will focus on comparing the expression of the heat shock protein 70 gene among sites. Previous studies had demonstrated that when *C. virginica* is exposed to organic contaminant and heavy metal the expression of the heat shock protein response (HSP70 family) is increased. It is known that HSP70 is a gene that codes for chaperone proteins that regulate protein folding when a cell is stressed. If there is misfolding in a protein, HSP70 would repair the protein by folding it into its native state. When an organism is undergoing stress from its environment, such as in elevated temperature, proteins are more prone to misfolding. This increase in misfolding promotes the production of HSP70 chaperone proteins to repair the misfolded proteins and will result in higher gene expression in the organism (Mayer et al. 2005). Under conditions of stress, the levels of HSP70 should be elevated in order to repair the damage. Since the Maine hatchery oysters are foreign to the estuaries of New York, we expected that these oysters may have a higher level of HSP70 gene expression than the wild type. If the results indicate that the wild oysters have a higher level of HSP70 gene expression,

then that suggests that Maine oysters could be used for oyster restoration.

Furthermore, it has been shown that the exposure of the eastern oyster suspended clay particles spiked with polynuclear aromatic hydrocarbons (PAHs) and to suspended field contaminated sediments (SFCS) induced the higher response of the heat shock proteins ((Boutet I, et al., n.d.). On the other hand, other studies conducted showed that the introduction of metal in the oyster's environment might also block the stress response of the organism. Oysters that were exposed to metal were found to have reduced expression of the HSP70 on the gills and digestive tract where exposed to metals such as copper and cadmium. ((Boutet I, et al., n.d.)). Because of its confirmed role in the stress response, HSP70 is a good choice as a starting point for exploring differential gene expression between wild and hatchery oysters in the Hudson River Estuary. Furthermore, we choose the gene Beta-Actin as our control since it is considered a housekeeping protein that is expected to show very little difference in gene expression among environments (Lupberger et al. 2002)

Methods

1. Sampling

Maine hatchery oysters were sampled , in a previous study done by Chelsi Napoli(Napoli, 2018),from Billion Oyster Project restoration cages, from two different sites in the New York Harbor, including Governors Island (2016 hatchery stock sampled in August 2017) and Bush Terminal (2015 hatchery stock sampled in September 2017). We sampled wild oysters from the shores of Soundview Park (July 2018). Approximately 10 g of gill tissues were taken and stored in RNAlater at -80°C for stabilization. The sample size consisted of 20 oysters per site. It is important to note that the methods below were applied only to oysters from Soundview Park and the results were compared with the ones in the previous study.

2. RNA extraction and synthesis of cDNA

RNA was isolated from the oyster tissues using the ZYMO Quick-RNA Mini kit. Before using the kit the tissues were crushed using RNase-free pestles and incubated at 55°C in proteinase K for ~one hour. We followed the ZYMO protocol but increased the quantity of Lysis Buffer from 300 μl to 600 μl. The extracted RNA was quantified using the Nanodrop in order to know the concentration and purity of the sample. Furthermore, the RNA was treated with the Invitrogen Turbo DNA-free kit to remove any genomic DNA contamination. We added 3 μL of TURBO DNase enzyme to 45μl of RNA. Afterward, we added 5 μl of 10X Turbo DNase buffer and followed the kit manual instructions from Invitrogen TURBO DNA-free user guide.

The RNA was used to make cDNA using the Bio-Rad iScript Reverse Transcription Supermix kit. The oyster cDNA was quantified with the Nanodrop in order to know the level of concentration and purity of the sample, which was given by the ratio of A260/A280. We expected the obtained A260/A280 ratio to be about 1.80 in order to be considered suitable for analysis. Values lower than that would be an indicator of contamination. After obtaining the concentrations of the nucleic acid, dilutions were prepared by using the formula C1V1=C2V2. This was done to calculate the concentration of water and cDNA needed.

3. **Quantitative polymerase chain reaction**

The gene Beta-Actin was selected as our control, and our gene of interest was HSP70. Primers were designed for both genes in a previous study conducted by Chelsi Napoli, who consulted with Tara Ellison (BIO-RAD Laboratories) and the MIQE guidelines (Bustin et al., 2009), in order to determine the optimal qPCR conditions. (Napoli, 2018). They estimated that the optimal annealing temperature for the CFX96 system is 60°C(BIO-RAD Laboratories). The primers were designed using the default settings of Primer3 and GenBank accession AJ271444.1 (Untergasser et al., 2007). Additionally, they checked for alignment with the sequence Clustal Omega (McWilliam et al., 2013). The forward primer for HSP70 was 5'-AGCCAGATTTGAGGAGCTGT-3' and the reserves primer was 5'-TTGTCTAGTTTGGCGTCCCT-3'. The designed primers consisted of an 85 bp long amplicon between 1039 and 1125 bp in the sequence (Napoli 2018). Moreover, the -
-actin primers were designed with the use of Primer3Plus (Untergasser et al., 2007),

using the gene sequence from GenBank accession number X75894.1 as a template.

The sequences for forward and reverse primers, respectively, are Beta-

Actin_Cv_Dec17_F 5'-GTACTGTCCCTGTACGCTTC-3' and Beta-Actin_Cv_Dec17_R 5'-

CTCCGGAGTCGAGTACGATA-3'(Napoli.,2018). The annealing temperature for the

primer of both Beta-Actin and HSP70 was determined to be 60.0°C and validated

with the software, PrimerBLAST (Ye et al., 2012;Napoli 2018).

Previously two sequences for the gene Beta-Actin were published in GenBank,

therefore in order to generate the new primers, the two sequences were checked for

alignment using Clustal Omega (McWilliam et al., 2013). The Genbank accession

numbers were: GenBank accession number X75894.1- and GenBank accession

number CF646509.1 (Napoli et.al,2018). Furthermore, we used 0.2 ml low profile

thin-walled 8 tube and ultra clear cap strips from Thermo Scientific, as well as Bio-

Rad Hard-Shell 96-well thin-wall PCR Plates.

　　　We proceeded to perform polymerase chain reaction for both genes, using

the cDNA that we synthesized. Negative controls were included for each run (as

below). We also performed serial dilutions by pipetting 1 ul of cDNA into a test tube

and then diluting that 1 ul of cDNA with 9 ul of water. 1 ul of the diluted sample was

extracted and pipetted into a different test tube and then diluted again with 9 ul of

water. This serial dilution process was repeated 3 times for each sample for both

genes. This serial dilution was performed to test the efficiency and R2 values for

each primer pair. The efficiency value was about 140.2%, which was acceptable

since we were expecting a value of 100% since the theoretical maximum of 1.00 (or

100%) indicates that the amount of product doubles with each cycle (MIQE, 2015).

On the other side, the R^2 value was 0.718. However, there were several errors with the PCR, and that is somewhat reflected in the high value of efficiency . This suggest that the amplicons were more than doubling each cycle. This means that primers were annealing to more than just the cDNA template. Moreover, the low R^2 value indicates that there were errors during pipetting the same amount of nuclei acid into each of the tripiclate well

To conduct the PCR, we used cDNA that was diluted to 100 ng/μl. In strip tubes of around 0.2 ml and ultra clear cap strips from Thermo Scientific were use to cover, as well as Bio-Rad Hard-Shell 96-well thin-wall PCR Plates , we pipetted 1μl of cDNA, 1μl of Forward primer(10 uM, suspended in water), 1μl of Reverse primer (10uM, suspended in water), 7μl of nuclease-free water, and 10μl of Bio-Rad iTaq Universal SYBR Green Supermix. We performed PCR in triplicate using the cDNA for each individual oyster sampled. For each PCR run, we also performed a negative control using oyster RNA samples that underwent a no reverse transcription protocol while the oyster RNA samples underwent a reverse transcription protocol. We originally ran the PCR for 40 cycles but had changed the PCR protocol to 50 cycles for heat shock protein to obtain a more ideal sigmoidal curve, in order to allow HSP70 to reflect a Cq value and display a sigmoidal curvature. The results were obtained using Real-Time PCR (qPCR) CFX96 and the Bio-Rad CFX Manager system.

Furthermore, when looking at the results from the RT-PCR, one can observe that the samples of cDNA cross the threshold at similar cycles as the controls, which was not expected at all. Since polymerase chain reaction is sensitive to the quality and quantity of

DNA, we were expecting that our samples contained more nucleic acid and would cross at earlier cycles in comparison to the controls.

4. **Statistical Analysis:**

We conducted a statistical analysis of the data using the R program (3.3.2 version). Our first step was to calculate the average Ct value for each gene for each oyster sample, averaged across the three runs. The Ct value is the number of PCR cycles that occur until the relative fluorescent units cross a built-in threshold value. Next, normalized Ct values were calculated by taking the ratio between the average Ct values of HSP70 and the average Ct values corresponding to Beta-actin. The assumptions of the ANOVA were violated because the Soundview site had a higher variance in normalized gene expression values than the other two sites as we can observe in Table 1. Thus, we conducted a Kruskal-Wallis rank-based test to compare the medians among the three sites. Because the Kruskal-Wallis cannot tell us which sites differ from each other, an ANOVA analysis was conducted, followed by a Tukey-Kramer test to observe which site differed from the others. Results of the normalized Ct values are displayed in a strip chart and the ANOVA observations in a table.

Results

In Figure 1 we could see that variance were similar among the group of oyster in Bush Terminal and Governors Island, but differ significantly from the oysters from Soundview Park. Moreover, in Table 1 mean, the standard deviation for each group were calculated, being the wild oysters from Soundview Park the ones that showed the highest standard deviation and highest mean as well. In addition, in Table 2 the results from the Kruskal-Wallis test, showing that there is a real difference among groups , and in Table 3 and 4, the results of the ANOVA and Tukey-Kramer test, are shown, indicating that the oysters from Soundview Park were the ones that differ significantly from the other group.
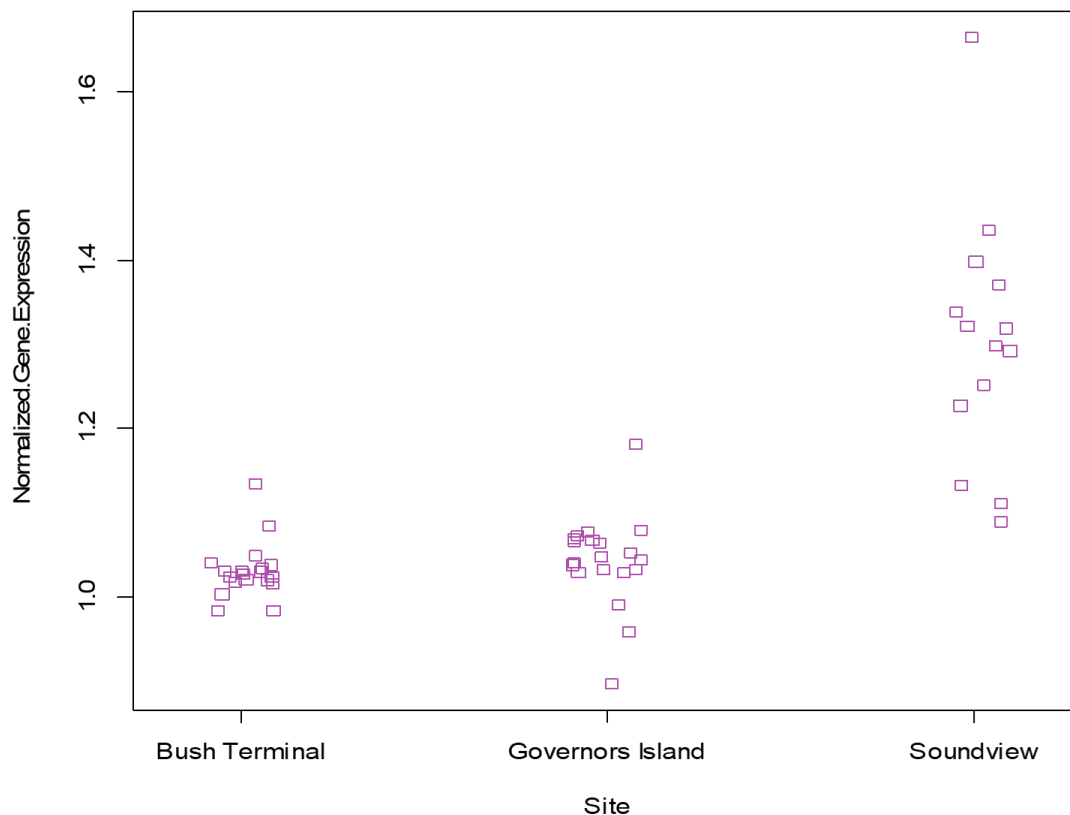
Figure 1.Stipchart of Normalized Gene Expression per site. Normalized gene expression is the average CT values for HSP70 divided by the average CT values for Beta-Actin for each oyster.

Table1. Calculated mean and standard deviations for normalized gene expression for each three sites

Site	Mean	Standard deviation
Governors Island	1.043671	0.05451509
Bush Terminal	1.031241	0.03347013
Soundview Park	1.303357	0.1477316

Table 2. Results from the Kruskal-Wallis rank-based test to test for differences in median normalized gene expression values among the three sites.

Kruskal-Wallis test statistic	Degrees of freedom	P-value
31.43	2	1.496x10^-07

Table 3. Results after conducting ANOVA analysis using the program R (version 3.3.2). The response variable were the variance among sites and within oysters strains(wild or from hatcheries) and the explanatory variables was the site and oyster strain

	Degrees of freedom	F value	P value
Mean square group	2	50.58	<0.0001
Mean square error	50		

Table 4. Tukey-Kramer Results using R program (version 3.3.2), variances among group of oyster from each site were compared to observed which one differ significantly from the other. Soundview Park appear to be the most significantly different from the others.

	Estimate	Std. Error	t values	Pr(>[t})
Governors Island –Bush Terminal	0.01243	0.02720	0.457	0.891
Soundview - Bush Terminal	0.27212	0.02990	9.100	<0.001
Soundview - Governors Island	0.25969	0.02958	8.778	<0.001

After conducting an ANOVA analysis using the R program, one can conclude that indeed there is a difference between sites, as expected. This conclusion is supported by our results from the Kruskal-Wallis test and ANOVA, which suggest that at least one of the group differs significantly from the others. The F value represents the ratio between the variance among groups divided by the variance within groups, by having a critical F value lower than our calculated F value we reject the null hypothesis; moreover, the p-value obtained was of <0.001, which is lower than our alpha value of 0.05, indicating that there is a significant statistical difference. However, this experiment should be repeated in order to have more accurate results and taking a larger sample size. In addition, we performed a Tukey-Kramer test in order to observe which sites differ from the others. We observe that Soundview Park differs significantly from the others two sites, this was somewhat expected since the oysters of Soundview are wild oysters, while the oysters that were sampled from Governors Island and Bush terminal were from hatcheries. This gives us insight that either genetic variation or the influence of early rearing conditions in hatcheries is more important than the environmental factors in shaping contemporary responses to the environment via heat shock protein. .

Discussion:

We decided to sample oysters, including wild oysters and oysters from Billion Oyster Project restoration cages, from three different sites of the New York Harbor, where conditions were varied: Governors Island, Bush Terminal, and Soundview Park. Additionally, we chose to focus on the genes Beta-actin, since it is a positive control that mainly all organism express and our gene of interest is Heat Shock protein 70, which we know expresses under stressful conditions.

We previously hypothesis that similar responses between wild and hatchery oysters would tell us that the hatchery stock is a good choice to use in restoration programs. In the future, additional students working with Dr. Crispo will study additional hatchery stocks to help elucidate which oyster stocks respond most appropriately, with the least amount of stress, and most similar to wild oysters. This work will help inform oyster restoration biology. Oysters, as mentioned above, are considered foundational species that engineer the environment, and their decline could affect the balance of the whole marine ecosystem, and thus their restoration efforts are of paramount importance. Including both strains of oyster from each site so that will have a paired design required to test our hypothesis. This would of extreme importance since by doing that we are controlling the environmental factors and we would be more certain that differences in response would account mainly to the genetic component, which is the of our interest. This experiment tells us whether the genetic expression shows a better coping mechanism to an environmental factor or the other way around. As mentioned before if both oysters

strains show similar gene expression levels, therefore, we can use the oysters from hatcheries for restoration purposes.

It is known that in marine environments, natural stressors often interact in complex ways leading to a 'stress landscape' that demands physiological responses (Chapman et.al 2011) In the recent past, this landscape has been further complicated by anthropogenic inputs, and it requires the organism to undergo acclimatization of adaptation in order to survive. Previous studies have studied, trough microarrays, the mRNA expression of genes in response to environmental factors and to test the level in which the environment can modify the genetic expression. Some of the genes known to be activated under stressful conditions are metallothioneins, glutathione-S-transferase, heat shock proteins, and cytochrome P-450, Moreover number of genes involved in protein stabilization(including chaperonins and heat shock proteins, or Hsps) and DNA stabilization (histones) are found to be discriminatory factors of stress. Environmental factors that were found to have the biggest effect in gene expression patterns were the pH and temperature (Chapman et al, 2011). In the study of Chapman et al. (2011) it was discovered that Hsp70 is up-regulated with increasing temperature and decreasing pH. Furthermore, when interpreting transcriptomic data we typically assume that changes in the mRNA expression translate into changes in the proteome and subsequently metabolic processes; recently as shown by (Newman et. al.2006) in yeast 87% of the protein levels were correlated with their mRNA expression levels. This is one of the reasons why we look at nucleic acid concentration.

The data in Chapman et al. (2011) showed that there was a shift in Beta-actin expression in response to pollutants, suggesting that-actin used for baseline corrections in qPCR in previous studies (Ivanina et al.2008) and references that it may not be always reliable as a housekeeping gene. However, the shift in actin baseline would make the conclusions about the relative expression of other genes standardized to Beta-actin more conservative. We have to take this into consideration, different genes should be look in the future for further analysis. Moreover, as (Ivanina et al.2008) suggests about the transcriptional response of stress proteins to metal challenge that gene expression in hepatopancreas is a more reliable predictor of environmental stress than expression levels in gill, we should take this into account since we used gill tissues.

On the other hand, it is known that oysters contain high morphological plasticity as adults, but low variability in morphology as larvae. Plasticity allows the species to persist and survive in a highly variable estuarine environment (Eierman & Hare, 2015). Previous studies have analyzed how gene expression varied in response to changes in environmental factors. One of the research studies that has been done studied gene expression in oysters from low and high salinity, place them in a single estuary with salinity acclimation, and analyzed it in order to understand the relationship between plasticity and evolutionary processes using next-generation RNA sequencing technology. Indeed they found that the oysters had significantly different expression in response to salinity treatments (Eierman & Hare, 2015). This result suggests that oysters might have the ability to acclimate to their environments, and thus the stock origin would be of little importance to the survival and growth of the oysters. Therefore this would indicate that

oysters from hatcheries could be potentially used for restoration if they are able to adapt and cope well with the environmental conditions of the wild oyster.

After analyzing the data we can observe that the highest difference in variance was observed between oysters from Soundview park and oysters from the two other sites. This was expected since the oysters from Governors Island and Bush Terminal are from hatcheries and the oysters from Soundview were from the only ones from the wild. This gives us insight that perhaps the genetic expression to be more relevant than environmental factors , because even though Bush Terminal and Governors Island have different environmental conditions the oysters which were from the same strain behave in a similar way, therefore the strain was a more determinant factor than the environmental condition in terms of genetic expression. The next steps as mentioned before would be to collect both wild and hatchery oysters from the same site in order to control for different environmental conditions. Although the oysters showed differential gene expression, on average, form the hatchery we have to keep in mind that they also come from a different location, therefore is not possible to disentangle the effects of origin form the effects of the environment.

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