**Intro**

*Crassostrea gigas,* also known as Pacific Oyster, is an estuarine specie found in intertidal and sub tidal zones. The Pacific oysters are frequently undergoing stressors from the environment such as heat stress, toxins in the water, bacteria, ~~etc~~ [1]. Based off their physical anatomy, the hard outer shell acts as one defense mechanism against foreign intruders. Inside the shell, they have only an innate immune system. This acts by specialized immune cells recognizing and tagging foreign invaders and then self-ingesting to internally destroy it[1].

The objective of this research is to see if an immune response can be created in Crassostrea Gigas from the exposure of Beta-Cyclodextrin. The immune response will be measured by expression of three immune related genes: EP4 receptor for prostaglandin E, astacin and cystatin B. These are three immune related genes that all serve different functions [REF].

Cyclodextrin is a compound that is composed of sugar molecules bound in a ring like structure. It is easily made from starch and has been seen to have positive effects on immune systems of certain fish[3]. This compound is very water soluble based on its structure and also has the unique ability of forming host guest complexes [2]. Cyclodextrin can stabilize unstable compounds. Inclusion of certain compounds with cyclodextrin have been seen to penetrate tissue and can release biologically active compounds under certain conditions [2].

EP4 receptor for prostaglandin E is related to the G coupled protein family, which is used in signal transduction. Prostaglandin can bind to this receptor and trigger an activation in immune response [4]. They can also mediate growth, which is important when foreign invaders are present [REF].

Astacin is a gene that encodes for a protein family that includes bone morphogenetic protein (BMP-1) that helps in the formation of extracellular matrix [REF]. Another function of BMP-1 is the activation of latent complexes, which act as the secretion and folding element of transforming growth factor [REF]. It also has to roll of cleavage of proteins, specifically prolactin, which helps balance salt concentration in fish [REF]. By cleavage of this protein, angiogenic factors form, which is just a group of substances present in circulation.

Cystatin B is a gene that has been seen to increase due to immune compromised oysters in past experiments [REF]. It codes for a protein called Cystatin B and is thought to play a role as a protease inhibitor. It inhibits proteases from breaking down proteins that have been leaking from the lysosome that could be potentially harmful for the cell to reintroduce [REF]. The enzyme that is reduced in activating is called cathepsins. Overall, this protein helps to protect other proteins within the cell from harmful leakage of the lysosome.

Expssing *Crassostrea gigas* to beta cyclodextrin we provide insight into if oysters illicit an immune response similar to those observed in other teleosts. Measuring expression of PGER, Cystatin B, and Astacin will give an insight to what some of these immune related genes are doing when exposed for 24 hours to the beta-cyclodextrin.

**Methods**

All of the oysters for this experiment were obtained from Taylor Shellfish on Capitol Hill, which farms their oysters from the Hood Canal region of the Puget Sound. A control group of Pacific Oysters (15) at ocean like temperatures (around 50 degrees Celsius) were placed into a tank with a pump to induce water flow and also had an O2 pump to make sure there is a proper amount of oxygen supply to the oysters. These 15 oysters will act as the control group because they experienced no stress and had the same conditions as the tank they came from.

The experimental group had similar conditions with the same size tank and same amount of water (8L). It also had the same pump for water flow and O2 pump for proper oxygen supply. Prior to adding the last liter into the tank (before oysters were introduced), 1 liter was mixed with 2.009 grams of beta-Cyclodextrin. Stirring the powder substance for 5 minutes solubilized in the water. The 1 liter of beta-cyclodextrin water was then introduced into the experimental tank and let the flow pump work for about 5 minutes to make sure the cyclodextrin was mixed into the water. Another 15 Pacific Oysters were introduced into the experimental tank at 1:30p.m. on October 22, 2012 and allowed to bath for 24 hours.

On October 23, 2012 at 1:30p.m. the dissection process began. Length and width were measured (Length was hinge to farthest point, width was widest point). The oysters were then shucked and 2 gill samples and 1 mantel sample were dissected out and placed into tubes with its corresponding sample number. These were all frozen and saved for further processing.

Next, RNA from each of the samples was isolated using TriReagent and then quantified using a spectrometer to ensure there was the proper amount to make cDNA. Once determining proper RNA amounts, cDNA samples for each of the samples were created using thermocycling and adding A,C,T,G’s. During this process, primers were created for the three genes:

|  |  |
| --- | --- |
| Primer Name | Primer sequence |
| pger\_ep4\_fwd | ACCGAGAGTGCTGAGTGGTT |
| pger\_ep4\_rvs | GGCAAACTGTAAGCCAGGAG |
| astacin\_fwd | ACGCCCTAGTTGGATGAATG |
| astacin\_rvs | ACTTGGTCTGGGGTTGTTTG |
| cystatinB\_fwd | GAGATTCCCCCTCACTCCTC |
| cystatinB\_rvs | TGCTGAAAGCCTCCAAATCT |

After primers had been designed and rehydrated a test qPCR run was administered, using sample immune compromised tissue from Pacific Oysters from the Roberts Lab. After getting the test results back, only astacin and cystatin B were decided to continue with the experiment considering the pger\_ep4 primer was not detected.

Lastly a qPCR was ran for the astacin and cystatin B genes using the cDNA from the control and experimental oysters under these parameters: 1. 95°C for 10 minutes 2. 95°C for 15s 3. 55 °C for 15 s 4. 72°C for 30 s (+ plate read) 5. Return to step 2 39 more times 6. 95°C for 10s 7. Melt curve from 65°C to 95°C, at 0.5°C for 5s (+plate read). Data was then exported and ran through PCR miner and compared to a normalizing gene (elongation factor) for overall gene expression.

**Results**

To test the hypothesis, gene expression was evaluated for PGER, Cystatin B, and Astacin genes. PGER gene didn’t make it past the trial qPCR run due to and primer dimer occurring. When running the qPCR for Cystatin B and Astacin, only Astacin gene gave data that was worth looking at. The Cystatin B data had multiple melting points in not only the experimental but the control group, as well as showing primer dimer in the blank samples closer to the later cycles. We came to the conclusion that the data would not provide any accurate information and therefore was disregarded.

This left us with the PCR for the astacin gene. After exporting the data from the qPCR, it was placed into PCR Miner, which gave us CT, Gene efficiency, and much more usual data. From this data an initial gene expression was found from the formula: 1/(1+average efficiency)^CT. After these were calculated seperatly for both the control and experimental groups, it was then divided by a normalizing gene, elongation factor 1 alpha, which had close to no change between the two groups. This gave us the overall gene expression for each sample (unitless).

Next a two tailed t-test was ran comparing the control to the experimental group and gave a p-value of 0.525. This suggests that the data was not significant. By looking at the graph of expression before comparing to the normalizing gene (fig 3), less variance is seen. A two tailed t-test was then ran for the normalizing gene to ensure that it had nothing wrong. The results had a p-value of 0.001.

*Figure 1:* This graph represents the difference in gene expression from the control and experimental groups averages. It also shows the standard deviation pictured as standard error bars. There is a slight increase in overall gene expression after being exposed to the cyclodextrin, but the significance is quite low (P-value: 0.525). *Figure 2:* This graph represents the data as individual gene expression rather than averages. Astacin: control group, AstacinE: experimental group. Overall, a lot of variance is seen between the control and experimental groups. These gene expression are based off a normalizing gene (elongation factor) which doesn’t change from stress. From the graph there is no clear outliers or any data that suggests that certain samples were affecting the rest of the data.

*Figure 3:*  This graph represents the gene expression before comparing it to the normalizing gene. Astacin: control group and AstacinE: experimental group. This brings up some interesting data in the sense that there is a large increase in overall gene expression for the astacin gene. This could bring up issues with the normalizing gene.

**Disscusion**

The technology and biochemistry behind beta-cyclodextrin has been around since the late 1970’s [REF]. The compound is used in numerous pharmaceutical drugs for its ability to act as a water soluble carrier [REF]. Also, the drug has been used to architect more complex molecules due to its unique ring shape structure. Recently, studies have shown that not only is the cyclodextrin molecule a good water soluble carrier but has strong effects itself. In humans that had Niemann Pick C diseas, a disease that effects the metabolism of cholesterol, cyclodextrin was administered do to it being bulky and hydrophobic to cholesterol [REF]. This was put directly into the blood brain barrier for maximum circulation[REF].

For most of the studies surrounding the clinical use of cyclodextrin, it has been used as a way to carry other molecules, or in nutrition, bring in better nutrients [REF]. As seen in the data on figure 1, there was no significant change in the amount of astacin being expressed before or after the cyclodextrin was in the system for the 24 hour period. The thought process behind this gene was the cyclodextrin being a bulky hydrophobic molecule, would change the formation of some of the cytoskeleton within the cell and cell membrane. Astacin, a protein that helps to build and rearrange cytoskeleton, would be unregulated due to this difference in cholesterol. This suggests that the cyclodextrin does not have a direct effect on the gene expression of Astacin by itself.

In most of the studies surround cyclodextrin, it has been treated with other nutrients, molecules or something that the cyclodextrin complex is carrying. Not much research has actually looked at the cyclodextrin molecule by itself. This brings up the discussion that if a pathogen or some foreign molecule was present while the oyster also had the cyclodextrin present, if it could lead to any change in gene expression. A study on zebra fish showed that cyclodextrin bounded to C60 fullerene, a supplement that is said to increase length in lifespan, produced an immune response, but when tested again without the supplement, the same immune response was seen. In the study in oysters by only introducing the cyclodextrin and no form of pathogens, no immune response will be seen.

By not seeing an immune response doesn’t mean that the molecule won’t work in the presence of a threat. It could be that during a threat, the molecule might help increase signals being sent from cell to cell and increase an immune response in this regard. Also, the gene Astacin, which codes for a protein that helps rearrange the cytoskeleton, might not have any function until an immune response it needed. Compared to these other studies where immune response is seen to occur, there is some form of bacteria or pathogen present, which might be a form of inducing it; because not only was there an increase in immune activity, but also a lower mortality rate. This is a key component to the cyclodextrin acting as an immune stimulant.

The data in both figure 1 and figure 2 show that there is a wide range of variance in all the samples. It was interesting to see how there was large amounts of astacin in the last three samples in the control while others were expressing close to zero. This could have been a result of prior contamination when receiving the oysters or some other immune compromised stress they had undergone.

Clearly the data shows how there is no real correlation between cyclodextrin sample and the control group. This does leave room for further research into other known immune cell related genes that have been up regulated due to a pathogen. Also, testing to see if cyclodextrin was introduced with the C60 fullerene just like the zebra fish in order to get an immune response would be something to look into.

It would be beneficial to keep looking into ways to stimulate an immune response in the pacific oyster for increased overall health. Invertebrates have not had much research done apart from identifying these immune related genes. By figuring out how the physiology behind boosting an immune response, it may lead to further explanations about more complex systems (eg. Humans) and also how natural selection has played a role.

**Resources:**

[1] Provider: John Wiley & Sons, Ltd

Content:text/plain; charset="UTF-8"

TY - JOUR, AU - David, Elise, AU - Tanguy, Arnaud, AU - Pichavant, Karine, AU - Moraga, Dario, TI - Response of the Pacific oyster Crassostrea gigas to hypoxia exposure under experimental conditions, JO - FEBS Journal, VL - 272, IS - 21, PB - Blackwell Science Ltd, SN - 1742-4658, UR -, <http://dx.doi.org/10.1111/j.1742-4658.2005.04960.x>, DO - 10.1111/j.1742-4658.2005.04960.x, SP - 5635, EP - 5652, KW - Crassostrea gigas, KW - hypoxia, KW - suppression subtractive hybridization libraries, KW - gene expression, PY - 2005

[2] Provider: John Wiley & Sons, Ltd

Content:text/plain; charset="UTF-8"

TY - JOUR, AU - Saenger, Wolfram, TI - Cyclodextrin Inclusion Compounds in Research and Industry, JO - Angewandte Chemie International Edition in English, JA - Angew. Chem. Int. Ed. Engl., VL - 19, IS - 5, PB - Hüthig & Wepf Verlag, SN - 1521-3773, UR - <http://dx.doi.org/10.1002/anie.198003441>, DO - 10.1002/anie.198003441, SP - 344, EP - 362, KW - Cyclodextrins, KW - Inclusion compounds, PY - 1980, ER -

[3] Cyclodextrin on fish paper (Need to figure out how to cite)

[4] Roberts, S, G Goetz, S Whites, and F Goetz. (2008): n. page. Web. 8 Nov. 2012. <http://www.ncbi.nlm.nih.gov/pubmed/18622569>.