USING MOLECULAR BIOMARKERS TO DETERMINE POSSIBLE EFFECTS OF SHELLFISH AQUACULTURE ON NATIVE POPULATIONS OF *CRASSOSTREA VIRGINICA*

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ABSTRACT

Oysters are a keystone ecological species that generate numerous direct and indirect ecosystem services. They are also an important species economically, generating over 150 million dollars in 2014, which has led to the rapid expansion of aquaculture facilities and sites throughout the world. However, oyster aquaculture reefs my pose specific and/or novel difficulties for native oyster populations in surrounding areas. The change in regulation of genes within the *Crassostrea virginica* genus relating to metabolism, immunity, and cellular stress can be used as indicators, or molecular biomarkers, to determine whether the oyster is being exposed to harmful biotic or abiotic factors. The aim of this study is to use these established biomarkers to determine if possible changes to wild oyster gene expression can be attributed to the presence of shellfish aquaculture operations. In a study conducted in Big Bay, North Carolina, the expression levels of 7 genes were used as biomarkers of stress (AS6, KCrec, EDL, HSP70, PRDX6, GS, and SUP) their expression levels were then analyzed using qPCR. Differences in gene expression between control and potentially impacted sites, in gill tissue, was found for the EDL, HSP70, PRDX6 and GS genes. Differences in gene expression between seasons, in gill tissue, was found for the AS6, and KCrec genes. Body tissue was less sensitive, with only two genes showing differential expression (PRDX6 and SUP). This research shows that there is a difference in gene expression between naturally occurring reefs that are closer to aquaculture sites and naturally occurring reefs that are farther from aquaculture sites, supporting their continued use as molecular biomarkers of stress in oysters.

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1. **INTRODUCTION**

1.1 *Aquaculture*

The harvest of cultured fish, crustaceans, and mollusks has risen dramatically since the 1980's and is the fastest growing food producing sector in the world (Cranford et al., 2012). As the demand for seafood products continues to expand it is unlikely that the harvesting of fish from collapsing wild stock can be increased significantly, therefore in many cases aquaculture is the only way to increase the amount of protein available from seafood (Byron, Jin, & Dalton, 2015). In fact, because fisheries are in decline due to decades of overfishing, changing climate, and negative effects from human development, there is increased pressure on coastal and estuarine communities to expand and enhance seafood protein aquaculture (Burbridge, Hendrick, Roth, & Rosenthal, 2001). Given that saltwater environments offers the largest available area, it is anticipated that in the coming years the principal growth in aquaculture will be in those species that can inhabit and thrive in marine waters and estuaries (NRC, 2010). Bivalve aquaculture in particular is already a rapidly growing, global industry that occurs primarily in coastal waters and depends on functioning, productive ecosystems (FAO, 2006; Ferriss, Reum, McDonald, Farrell, & Harvey, 2016). Aquaculture development faces many challenges in the coming years as more focus is placed on environmental impacts of aquaculture facilities on native species, competition for water resources intensifies, and demand for aquaculture product increases with a growing population (Burbridge et al., 2001; Cranford et al., 2012). Although the potential benefits associated with sustainable coastal aquaculture are significant, there can also be significant drawbacks (S. Beck & La Peyre, 2015; Byron et al., 2015; Carmichael, Walton, & Clark, 2012; Ferriss et al., 2016; Forrest & Creese, 2006; Forrest, Keeley, Hopkins, Webb, & Clement, 2009), and public attention tends to focus on those species that are notoriously

environmentally inefficient or disruptive such as salmon and shrimp (Hopkins et al., 1995 Chopin et al., 2001; Neori et al. 2004). Uncontrolled effluent from shrimp farms causes eutrophication of the surrounding environment and salmon draw on the environment because they must be fed other fish (Dumbauld, Ruesink, & Rumrill, 2009). Oysters however, feed on particulate matter already present in the environment and therefore do not require the growth and distribution of food, along with the fact that as oysters feed they clean the water via filtration (Burell, 1988). Bivalve shellfish aquaculture is a relatively new practice in the United States compared to its history in other countries, it began in the late 1800's along the West Coast and has moved along the East Coast from the Chesapeake Bay, into the Carolinas (Carmichael et al., 2012; Cerco & Noel, 2007; Dumbauld et al., 2009; Stone, Hadley, & Kingsley-Smith, 2013). While cultured seafood production has been increasing, there is still a gap between wild caught seafood supply and demand which explains the tremendous growth of the aquaculture industry in the last decade and into the foreseeable future (Chu, Anderson, Asche, & Tudur, 2010; FAO, 2016). Shellfish aquaculture is not only environmentally important but is also economically important, particularly to coastal communities, it has been shown that when operating at carrying capacity the output and employment figures for a bivalve aquaculture industry would be significantly higher than those of harvest fisheries or seafood processing (Byron et al., 2015; Reum et al., 2015). As the industry expands and coastal waters and estuaries are converted to shellfish farms, concerns have been raised about the positive and negative ecological effects of aquaculture on coastal environments and ecosystems (Cranford et al., 2012; Ferriss et al., 2016; Forrest et al., 2009; Reum et al., 2015).The future of bivalve aquaculture development and growth in the United States rests on sustainable maritime practices on the part of industry, along with more thorough and informed management and oversight. Shellfish aquaculture must be

included in future development plans for coastal land and waters (Burbridge et al., 2001; Dumbauld et al., 2009) and it is imperative that we understand the full impact of oyster aquaculture facilities on native oyster populations so that we have the information needed to ensure that best farming practices and beneficial oversight is available to the stakeholders and end users of aquaculture facilities and their products. Uncertainty about the possible ecological effects of estuarine shellfish aquaculture operations, both positive and negative, remain high (Coen et al., 2007; Reum et al., 2015). This research aims to ascertain whether existing aquaculture facilities are stressfully impacting native oyster populations

1.2 *Oysters as a model*

Crassostrea virginica is a sessile, filter feeding, marine bivalve that is native to the East Coast of the United States as well as the Gulf of Mexico (J. L. Zhang, Walton, & Wang, 2017) and is common to the intertidal zone of most estuaries in the southeastern United States (Dame, 1972). *C. virginica* spawning is intermittent from May-November, eggs and sperm are released in the water column, where fertilization occurs. Within 8-9 hours of fertilization trochophore larvae have formed and 12-16 hours after fertilization veliger larvae form, both stages are planktonic and the larvae float in the water column for up to three weeks. The final larval stage is pediveliger which is characterized by eye spots and a foot, enabling attachment to a substrate thus becoming a spat (Burell, 1988). Literature varies on the growth rate of *C. virginica,* with some studies indicating a year one size range of 20.0-37.3 mm and others indicating a range from 50-70 mm. Observed year one size differences may be due to different biotic and abiotic factors like growing temperatures, salinity, tidal range, and siltation (Munroe, Borsetti, Ashton-Alcox, & Bushek, 2017; Paynter, Politano, Lane, Allen, & Meritt, 2010). *C. virginica* consumes food by

drawing water over its gills, trapping phytoplankton and zooplankton which it then moves to its mouth for consumption.

1.3 *Possible benefits of aquaculture oysters*

Oysters provide many ecosystem services and play a major role in the functioning of estuarine ecosystems (Dame, 1972). Oysters not only remove suspended particulates from the water column as they feed, but also reduce eutrophication by removing (Grizzle et al., 2017; Higgins et al., 2013) excess nitrogen and phosphorus from the environment. Therefore the presence of healthy oysters may be used to abate eutrophication in the surrounding water (Bricker, Rice, & Bricker, 2014). Oysters can also enhance nutrient removal through feeding and repackaging of seston biomass into meat, shell, and bio deposits (Carmichael et al., 2012). Bivalve filter feeders like oysters play an important role in benthic-pelagic coupling by transferring particles and nutrients from the water column to the sediments in the form of bio deposits (Bayne & Hawkins, 1992; Jordan, 1989). Bio deposits from *C. virginica,* in the form of feces and suspended particles that have been taken in by the oyster, rejected as a food source, coated in mucus and then expelled from the bivalve without having traveled through the digestive system (pseudofeces), may also promote phytoplankton growth in periods of nitrogen limitation (Cranford et al., 2007). Oysters also improve the water quality through seston removal, increasing the amount of filtering organisms present will increase the amount of nutrients being filtered out (Froehlich, Gentry, & Halpern, 2017; Nelson, Leonard, Posey, Alphin, & Mallin, 2004), and clarifying the water column can encourage growth of benthic microalgae and seagrass which deepens the oxic zone by allowing more photosynthesis in the water column (Cerco & Noel, 2007; Newell & Koch, 2004; Wall, Nystrom, & Litten, 2008).

The reefs that oysters form, which would otherwise simply be bare sediment, provide yet another ecosystem service by increasing habitat complexity which can encourage the settlement of more bivalves (O'Beirn, Ross, & Luckenbach, 2004), as well as attract other invertebrates and recreationally and economically important fish species (Dealteris, Kilpatrick, & Rheault, 2004; Tallman & Forrester, 2007). Chronic habitat degradation via overfishing of the population, removal of habitat and shell substrate, and accumulation and input of organic and inorganic contaminants has led to a severe decline in oyster populations (Fitzgerald, 2013). The number and condition of oyster reefs is decreasing drastically, globally more than 85% of oyster reefs have been identified as functionally extinct (M. W. Beck et al., 2011). As remediation efforts have been undertaken to reestablish extinct reefs and resuscitate dying ones it has been shown that implementing shellfish aquaculture has the potential to aid the conservation effort (Froehlich et al., 2017). introducing more shellfish to an ecosystem via shellfish aquaculture operations transforms largely unstructured bottom to one that is dominated by artificial structure and shell which will attract associated flora and fauna (Coen et al., 2007; Coen, Luckenbach, & Breitburg, 1998; Dealteris et al., 2004).

1.4 *Possible drawbacks of aquaculture oysters*

Although the addition of shellfish aquaculture to an area may have positive effects on the surrounding ecosystem by increasing the capacity at which the oyster population can perform their many beneficial services, it is important to understand that there are potential negative impacts of these aquaculture operations on the natural oyster population. Many biotic and abiotic factors found within estuarine environments can affect the health of oysters, such as variations in salinity, temperature, siltation, population density, increased human contact and mechanical agitation due to husbandry and harvesting of shellfish, and water level (S. Beck & La Peyre,

2015; Cressman, Posey, Mallin, Leonard, & Alphin, 2003; Dumbauld et al., 2009; Ferriss et al., 2016; Forrest et al., 2009).

Because oysters are filter feeders they are subjected to an increased amount of bacterial, viral, and protozoan pathogens which could contribute to increased levels of stress in individual oysters or among an entire population (Forrest et al., 2009). Potential concerns for shellfish aquaculture include spreading of pests like *Cliona celata* and pea crabs (Carroll, O'Shaughnessy, Diedrich, & Finelli, 2015), spreading of diseases like *Haplospiridium nelsoni* (MSX), *Perkinsus* marinus (Dermo), and Roseovarius Oyster Disease (ROD) (Forrest et al., 2009; Proestou et al., 2016), Three of the most severe diseases affecting oysters are MSX, Dermo, and (ROD), these disease have been known to cause mass mortality events in large oyster popluations (Biancani, Carmichael, Daskin, Burkhardt, & Calci, 2012; Forrest et al., 2009; Genard et al., 2011; Piontkivska et al., 2011; Proestou et al., 2016). Parasitic and bacterial infections of oyster populations are thought to be exacerbated by anthropogenic stressors associated with over-crowding and stress from handling and husbandry (Kuchel, Nair, & Raftos, 2012). The higher abundance of oysters in an area could also lead to a reduction in available food sources for native oyster populations, natural and restored oyster reefs can cause localized depletion of seston as tidal currents pass over shellfish beds and large scale consumption of suspended particulate matter may also affect food quality by altering seston particle size and rations of particulate carbon and nitrogen (Cressman et al., 2003; Forrest et al., 2009; Grizzle, Greene, & Coen, 2008). This may lead the natural population to be more susceptible to microbial diseases since lack of a healthy diet can have a negative impact on oyster immune function (Hegaret et al., 2004).

Due to the fact that native Eastern oyster populations have suffered vast losses from, MSX, Dermo and ROD, oyster breeding programs along the East coast have been selecting for

oyster lines that possess resistance and/or tolerance to these three principal diseases (Proestou et al., 2016). The expanse of *Crassostrea virginica* aquaculture in recent years has led to an increase in the interest of engineering selectively bred oyster lines that are more resistant to these diseases and more efficient at reproduction and growth (Proestou et al., 2016). Eastern oysters used in aquaculture are typically triploid since they grow faster than their diploid counterparts (Walton et al., 2013) and many times are also genetically modified to be disease resistant (Peterson et al., 2009; J. L. Zhang et al., 2017). These modifications mean there is potential for the aquaculutred oysters to out compete and out survive the native oyster populations, due to the fact that they are genetically modified to be disease resistant, leading the modified oysters to become invasive, thereby diminishing the native oyster populations (Moehler, Wegner, Reise, & Jacobsen, 2011).

Increased hard surfaces like live and dead oysters, farm materials, and other structures from from aquaculture operations could also provide novel habitats for fouling organisms such as barnacles, bryozoans, and jellyfish polyps which would otherwise not occur (or be at reduced densities) in the abscene of the farms (Coen et al., 2007; Duarte et al., 2013; Dumbauld, Brooks, & Posey, 2001; Forrest et al., 2009). The historic role of the oyster industry in the global spread of non-indigenous species, biofouling pests, toxic or noxious microalgae, and disease is well-recognized (Forrest et al., 2009; Karatayev, Padilla, Minchin, Boltovskoy, & Burlakova, 2007; McKindsey, Landry, O'Beirn, & Davies, 2007) and could certainly be a cause for concern as it pertains to native oyster populations.

With the addition of aquaculture comes more human involvement and activity, causing native reefs to be exposed to more human activity and disturbances (Forrest & Creese, 2006; Forrest et al., 2009), i.e. dispersal and maintenance of gear, vessel movements such as propeller

wash, as well as harvesting of large amounts of oysters several times a year, (De Grave, Moore, & Burnell, 1998; Forrest & Creese, 2006). All the extra human activity and movement of bags and cages may lead to more mechanical and physical agitation of the sediment, water column, and the oysters themselves. Oysters have been shown to exhibit a stress response due to mechanical agitation (Lacoste, Malham, Cueff, & Poulet, 2001) and sediment burial or siltation levels can have lethal effects on native populations of *C. virginica* (Comeau, Mallet, Carver, Nadalini, & Tremblay, 2017). Sediment burial can be accelerated by mesh bags used in aquaculture, which can interfere with local hydrodynamics, reducing flow rates and promoting sedimentation (Comeau, 2014),

With an increase in oyster reef density there is also an increase in rates of oyster bio deposition, which can increases sedimentation rates (Grizzle et al., 2008; Higgins et al., 2013; Pietros & Rice, 2003), and may influence wild shellfish populations since excess suspended sediment can have negative impacts on oyster filtration, growth, and condition index (Page, Posey, & Alphin, *In Review*). Increased sediment organic content can change sediment redox conditions through microbial respiration of organic matter, this process can potentially decrease sediment and water column oxygen concentrations (Dahlback & Gunnarsson, 1981; Kaiser, Laing, Utting, & Burnell, 1998), this could increase physiological stress of cultured shellfish and adjacent wild populations (Kamphausen, Jensen, & Hawkins, 2011; Kennedy & Breisch, 1983; Powell, Klinck, Ashton-Alcox, Hofmann, & Morson, 2012). Aquaculture structures can also interact with flow dynamics, potentially affecting sediments (Comeau, 2014) and larvae. Physical disturbance and impediments found around an aquaculture site could lead to increased levels of stress in native oyster populations since there is the possibility that these factors could extend their effects to adjacent natural reefs (Dumbauld et al., 2009).

Oyster aquaculture reefs my pose specific and/or novel difficulties for native oyster populations in surrounding areas. An awareness of the health of native oyster populations and the stress that may be imposed on them by the presence of oyster aquaculture operations is valuable due to the fact that oysters are a keystone ecological species which generate a number of direct and indirect ecosystem services (Munroe et al., 2017) which are invaluable. There are many factors that can affect the health and viability of oysters and these factors have been well researched and documented, as have the effects of shellfish aquaculture on the ecosystem as a whole (Ferriss et al., 2016; Han et al., 2017), however research is still lacking in determining the direct or indirect stressors that the presence of aquaculture oyster operations may have on the native oyster population in particular. It is unknown what effects, if any, the introduction of shellfish aquaculture sites may have on surrounding native oyster populations.

It is clear based on previous research that healthy oysters are imperative to a properly functioning aquatic ecosystem and that oyster aquaculture sites are rapidly expanding throughout the United States (Ferriss et al., 2016; Munroe et al., 2017; Proestou et al., 2016). As such, knowledge and understanding of the effects that oyster aquaculture may have on the native population is of utmost importance to the health of all oysters and the waterways they inhabit.

2. GENE EXPRESSION

The change in regulation of genes within the *Crassostrea* genus relating to metabolism, immunity, and cellular stress can be used to indicate whether the oyster is being exposed to harmful biotic or abiotic factors (Genard et al., 2012; Matthew J. Jenny et al., 2002; Liu et al., 2017; Piontkivska et al., 2011). Responses to environmental disturbances associated with aquaculture, such as increased incidence of disease and stress, can be indicated by changes in gene expression (M. W. Beck et al., 2011; Lacoste, Malham, Gelebart, Cueff, & Poulet, 2002).

Eighteen genes have been identified as indicators of stress in oysters (Genard et al., 2012; Genard et al., 2011) and are involved in the above-mentioned groups of biological function; metabolism, immunity, and cellular stress and their change in regulation may be affected by a variety of factors. The following sections outline some specific factors that may cause stress in oysters, the genes those factors may affect, and whether we would expect to see up or down regulation of the genes if stressors such as, increased temperature, increased exposure to disease and pests, hypoxic conditions, and increase in pH etc. are present.

2.1 *Stress response*

Oxidative stress can be brought on by a variety of factors such as an increase in temperature, an increase in pH, or bacterial infection and has been shown to lead to an increase in the expression of antioxidant related genes like peroxiredoxin 6 (PRDX6), as well as heat shock protein 70 (HSP70) (Clark et al., 2013; Liu et al., 2017; Wang et al., 2012). Genes that code for proteins that participate in cytoprotective processes, such as pernin precursor and heat shock protein 70 have also been shown to increase upon introduction of the oyster to bacterial infection (Genard et al., 2013; Genard et al., 2012). Cytochrome p450 (CYP450) is another gene that codes for a stress response protein and has been shown to participate in xenobiotic detoxification (Campos, Tedesco, Vasconcelos, & Cristobal, 2012), if there were toxins present in the environment due to increased fouling organisms present on aquaculture gear, or an increase in the presence of toxicants such as heavy metals there would be an expected increase in the expression of the cytochrome p450 gene (Genard et al., 2012; Zanette, Goldstone, Bainy, & Stegeman, 2010; L. B. Zhang et al., 2012). Estuarine ecosystems receive a constant input of xenobiotics from urban areas, for example, polycyclic aromatic hydrocarbons, which have been shown to cause an increase in the gene expression of cytochrome p450 (Zacchi et al., 2017). Mechanical

disturbances can also cause a state of stress in oysters which may lead to an increase in the expression of general stress response genes. (Lacoste et al., 2002)

2.2 *Immune response*

If disease levels were to increase in the native population there would be an expected increase in the expression of genes relating to immunity and immune functions within the oyster (Genard et al., 2013; Genard et al., 2012). Cathepsin B (CTB) participates in pathogen recognition, if an oyster population is infected with a bacterial or viral pathogen, we would expect to see an increase in its transcription. Natural killer receptor (NKR) and Killer cell lectin like receptor (KCrec) are both part of the lectin receptor family, lectins are specialized proteins that can recognize specific pathogen associated carbohydrate structures and cause agglutination of cells, promote cellular adhesion, and mediate the innate immune response. (Ackerman & Iwama, 2001; Geijtenbeek, van Vliet, Engering, t Hart, & van Kooyk, 2004; McGreal, Martinez-Pomares, & Gordon, 2004; Yamaura, Takahashi, & Suzuki, 2008). We would expect to see an increase in the transcript number of both NKR and KCrec in an oyster under stress, indicating an increased immune response (Genard et al., 2012). RAS suppressor (SUP) functions as a cell division inhibitor, for an oyster living in a stressful environment we would expect to see an increase in the number of RAS suppressor transcripts.

2.3 *Metabolic response*

If oysters are experiencing a lack of oxygen, which could be due to a variety of factors such as heavy sedimentation, excess bio deposition, or algal blooms, they are able to undergo anaerobic respiration, however extended periods of sedimentation can lead to hypoxia (Comeau, 2014; Comeau et al., 2017; Hoellein, Zarnoch, & Grizzle, 2015). During times of anaerobic

respiration genes like malate dehydrogenase precursor, cytochrome C1 (CC1), and NADH dehydrogenase subunit 1, which are involved in the citric acid cycle and parts of the electron transport chain complexes I and III respectively, may be down regulated (Genard et al., 2012; Genard et al., 2011). Genes such as malate dehydrogenase, NADH dehydrogenase subunit 1, cytochrome C1, ATP synthase f0 subunit 6 (AS6), endothelial lipase (EDL), delta 9 desaturase, glutamine synthetase (GS), and translation initiation factor eIF-2B delta subunit are all related to energy metabolism within oysters (Genard et al., 2012; Genard et al., 2011). It has been shown that elevated temperatures as well as elevated levels of CO_2 and salinity can cause metabolic stress within oysters which would lead to a down regulation in genes like glutamine synthetase, cytochrome C1, and endothelial lipase (Genard et al., 2012; Ivanina et al., 2013; Piontkivska et al., 2011; Zacchi et al., 2017).

The specific aim of this study is to use previously established molecular biomarkers to determine if possible changes to wild oyster gene expression can be attributed to the presence of shellfish aquaculture operations, using Big Bay North Carolina as a model system. We hypothesize that aquaculture facilities in Big Bay will influence the expression of stress related genes in native populations of *Crassostrea virginica*.

3. **METHODS**

3.1 *Specimen selection and preservation*

At the time the oyster samples for this study were obtained, September 2016 to June 2018 there were two oyster aquaculture sites in operation in the North Carolina National Estuarine

Research Reserve (NCNEER) Big Bay, which is a small embayment located in Masonboro sound off the East coast of North Carolina. Four oyster reefs that lie in close proximity to the two aquaculture sites have been designated as potentially impacted sites and three reefs further removed from the current aquaculture sites have been designated as control sites. Each site was sampled at low tide quarterly, for two years. Seven to ten oysters were collected from each site. Vernier calipers were used to measure oyster length in millimeters, oysters collected ranged in size from approximately 50-70 mm so are considered to be from 1-3 years old (Grizzle et al., 2017). The body and gill of an oyster perform different functions and therefore may differentially express genes, for this reason the body and gills of each oyster specimen were separated onsite using dissecting tools and were immediately place in separate Nalgene liquid nitrogen safe vials and frozen in liquid nitrogen. Each subsample was stored at -80 °C until tissue homogenization and RNA extraction could be performed. It is important to note here that because of extenuating circumstances due to severe weather in Wilmington North Carolina in 2019, namely hurricane Florence, more than half of the samples collected for this study were damaged and unable to be used. Due to the small sample size it was difficult to determine whether the differences seen in the subsequent data analysis were due to proximity to aquaculture leases or if site location within the bay and season of collection were confounding variables effecting the results

3.2 *RNA extraction*

RNA was extracted from all tissue samples using the TRIzol reagent (Invitrogen, Carlsbad, CA), (Brander et al., 2016; Genard et al., 2012; M. J. Jenny et al., 2016). Each sample of gill and body tissue was homogenized individually by extracting the sample from its vial and placing it in a sterilized mortar containing liquid nitrogen, a pestle was then used to grind the

sample into a powdered homogenate. 30-50 mg of the homogenate was then placed into a 1.5 mL tube containing 1 mL of TRIzol reagent on ice, a small sterilized pestle was then used to grind the powdered tissue homogenate completely into the TRIzol. 200 µl of chloroform was added to the tube and it was shaken vigorously for 15-30 seconds upon which it was incubated at room temperature for 3 minutes. The sample was then centrifuged at 13,400 rpm for 15m at 4 °C using an Eppendorf Centrifuge 5430R. After centrifugation, 450 µL of the aqueous layer was transferred to a new 1.5 mL tube along with the same amount of isopropanol and the tube was inverted 4 times to mix. The tube was then centrifuged at 10,000 rmp for 15 minutes at 4 °C. After centrifugation the supernatant was removed from the tube leaving a pellet of RNA material behind, the sample was left at room temperature for 10 m to dry. 1 mL of 75% ethanol was then added to the tube and it was centrifuged at 10,000 rmp for 5 minutes, this rinse was performed 4 times adding fresh ethanol each time. RNA was then resuspended in 100 mL of RNase-free water and stored at -80 °C until qPCR could be performed. Total RNA concentration and 260/280 rations (accepted values > 1.6) was determined using a Nanodrop 2000 spectrophotometer and the integrity of the RNA was determined by gel electrophoresis on a 1% agarose gel and visualized on a Gel Doc™ XR + Gel Documentation system (BioRad, Hercules, CA, USA, see appendix).

3.3 *cDNA synthesis*

cDNA was synthesized following (Brander et al., 2016) with some modifications. 2 µg of total RNA were used for cDNA synthesis the total volume of each sample was brought up to 30 µL total with nuclease-free water. cDNA synthesis reactions were conducted by adding 12 µL of 5X First Strand Buffer (Qiagen), 3 µL 0.1 M DTT, 0.75 SuperScript III RT (Qiagen), 3 µL 10 mM dNTP, 4.5 µL nuclease free water, 6 µL random primers and 0.75 µL of RNaseOUT to

each sample to make a final reaction volume of 60 µL. Samples were incubated at 50 °C for 50 min, followed by 95 °C for 5 min to stop the reaction, and then held at 4 °C until they were moved to −20 °C for storage until used in the qPCR reactions.

3.4 *qPCR*

At the onset of this study 18 genes related to stress in oysters were selected as potential molecular biomarkers to assess via qPCR based on (Genard et al., 2012), after performing efficiencies on each primer set for all 18 potential biomarkers it was determined that there were 7 primer sets which met the efficiency requirements of 95% or greater, (Livak & Schmittgen, 2001; Pfaffl, 2001), characteristics for each of these genes including the 2 used as reference genes can be found in table 1. The two reference genes selected were chosen based on those used in previous literature and also met the minimum efficiency testing requirements of 95% or greater, mentioned above (Etschmann, Wilcken, Stoevesand, von der Schulenburg, & Sterner-Kock, 2006; Genard et al., 2012; Livak & Schmittgen, 2001; Radonic et al., 2004). Oligonucleotide primers for qPCR were based on those in outlined in (Genard et al., 2012) and were ordered through Eurofins Genomics LLC. The SYBR Green qPCR reactions used a 1:36 dilution of template cDNA and were performed on a QuantStudio 6 Real-Time qPCR machine (Thermo Fisher Scientific, Massachusetts USA) in 96 well plates, as outlined in (Brander et al., 2016) with minor changes. qPCR reactions consisted of 10µL SYBR Green Probe, 0.5 µL of forward and reverse primers, 8 µL of nuclease-free water, and 1 µL cDNA. Cycling conditions were 30 s at 98 °C, 15 s at 98 °C, 40x cycles of 30 s at 60 °C and analyzed using QuantStudio Design and Analysis Software v1.5.0. Data were normalized to the R18s and β-actin reference genes, R18s and β-actin were both determined to be stable across treatments using GeNorm (Etschmann et al., 2006; Radonic et al., 2004; Vandesompele et al., 2002).

3.5 *Statistical Analysis*

Among and between treatment differences in gene expression were tested using ANOVAs in JMP Pro 14 (SAS, UNCW, NC) with an $\alpha \leq 0.05$, followed by a Tukey-Kramer post-hoc test for pairwise differences and a Students-t Test for differences between two treatment groups. Principal components analysis (PCA) was also used to determine similarities among gene responses which was done by treating each replicate in each treatment (site or season) as an observation (rows) and the qPCR response for each gene as a variable (columns); PCA was performed using a correlation matrix in JMP Pro 14 (SAS, UNCW, NC).

4. **RESULTS**

4.1 *Quantitative PCR (qPCR)*

The responses of 7 biomarker genes were first broadly characterized in *Crassostrea virginica* body and gill tissue through principal components analysis (PCA) through the treatments of site (control and potentially impacted) and season (fall, spring, and summer). Groups of genes showing similar patterns of expression have been identified, as determined by clusters in the PCA. Following PCA differences in qPCR responses among and between sites and seasons were tested using ANOVA, followed by Tukey-Kramer or Students-t Test post-hoc tests, depending on the number of treatments.

4.2 *qPCR PCA body tissue*

The PCA characterizing oyster body tissue from all control sites (Figure 1 a.) shows six of the genes loaded strongly onto component 1, which described 50.6% of the variance in the dataset, while one of the genes (PRDX6) loaded more strongly onto component 2, which described 22.5% of the variance. The corresponding PCA (Figure 1 b.) characterizing body

tissue from all impacted sites, shows all 7 biomarkers loading more strongly onto component 1, which describes 32.4% of the variance in that dataset. The PCAs characterizing biomarker response in body tissue from each individual control site (Figure 2 a., b., and c.) shows that for control site 1 (C1) four of the genes loaded more strongly onto component 1, which described 42.4% of the variance in the dataset, while three of the genes loaded more strongly onto component 2, which described 27.1% of the variance (Figure 2 a.). For control site 2 (C2) six of the genes loaded more strongly onto component 1, which described 49.9% of the variance, while the remaining gene loaded more strongly onto component 2, which described 23.5% of the variance (Figure 2 b.). For control site 6 (C6) four of the genes loaded more strongly onto component 1, which described 56% of the variance of the dataset, while the remaining two genes loaded more strongly onto component 2, which described 26.4% of the variance (Figure 2 c.). PCAs characterizing body tissue from each individual impacted site (Figure 3 a., b., and c.) showed that for impacted site 1 (I1) four of the 7 biomarker genes loaded more strongly on component 1, which described 45.3% of the variance, while the remaining three genes loaded more strongly onto component 2, which describe 29.1% of the variance in the dataset (Figure 3 a.) The PCA for impacted site 2 (I2) showed that 6 of the genes loaded more strongly onto component 1, which described 42.4% of the variance, while the remaining 1 gene loaded more strongly onto component 2, which describe 25.2% of the variance (Figure 3 b.). The PCA for impacted site 4 (I4) showed that 5 of the genes loaded more strongly onto component 1, which described 46.8% of the variance, while 2 genes loaded more strongly onto component 2, which described 29.1% of the variance (Figure 3 c.). PCAs were run on body tissue obtained in fall, spring, and summer, Figure 4 a., b., and c. The PCA corresponding to fall (Figure 4 a.) shows that 6 of the genes loaded more strongly onto component 1, which described 47.4% of the

variance, while the remaining gene loaded more strongly onto component 2, which described 19.2% of the variance. The PCA corresponding to spring (Figure 4 b.) shows that 4 of the genes loaded more strongly onto component 1, which describes 33.3% of the variance, while 3 of the genes loaded more strongly onto component 2, which describes 28.4% of the variance of the dataset. The PCA corresponding to body tissue obtained in the summer (Figure 4 c.) shows that 6 of the genes loaded more strongly onto component 1, which described 50.3% of the variance, while the remaining gene loaded more strongly onto component 2, which described 21.4% of the variance in the dataset. For all PCA results refer to table 2.

4.3 *qPCR ANOVA body tissue*

PRDX6 Expression values (MEVS) were significantly different between control and impacted sites from the fall (ANOVA, Students-t test, *P=.0486*), Figure 17. Suggestive significance in expression values for PRDX6 were found between control and impacted sites in the spring (ANOVA, Students-t test, *P=.0831*), Figure 18. There was also a significant difference found in expression values of PRDX6 between the fall and summer seasons (ANOVA, Tukey-Kramer, *P=0.0284*), Figure 19. In a comparison between individual sites it was shown that expression values for the SUP biomarker were significantly different between I2 and all other sites overall (ANOVA, Tukey-Kramer, *P=.0083* C1; *P=.0095* C2; *P=.0365* C6; *P=.0268* I1; *P=.0046* I4) Figure 26. PRDX6 and SUP were the only genes out of the 7 biomarkers to show any significance or suggestive significance in the *Crassostrea virginica* body tissue samples. Each of the remaining genes, HSP70, AS6, EDL, KCrec, and GS, showed no significant difference in expression in oyster body tissue.

4.4 *qPCR PCA gill tissue*

The PCA characterizing oyster gill tissue from all control sites (Figure 5 a.) shows that all genes loaded more strongly onto component 1 which described 55.3% of the variance in the dataset. The corresponding PCA characterizing gill tissue from all impacted sites (Figure 5 b.) all 7 genes loaded more strongly onto component 1, which described 45.2% of the variation of the dataset. Figure 6 a., b., and c. are PCAs characterizing gill tissue from each individual control site, C1, C2, and C6 respectively. Regarding site C1 (figure 6 a.) shows that 6 of the genes loaded more strongly onto component 1, which described 55.8% of the variance, while 1 gene loaded more strongly onto component 2 which described 19.2% of the variance of the dataset. PCA characterizing site C2 (Figure 6 b.) shows that 4 genes loaded more strongly onto component 1, which described 40.3% of the variation, while the 3 of the genes loaded more strongly onto component 2, which described 29.6% of the variation of that dataset. The PCA characterizing tissue from site C6 (Figure 6 c.) shows that all 7 genes loaded more strongly onto component 1, which described 73.6% of the variation in the dataset. Figure 7 shows PCAs characterizing gill tissue from each individual impacted sited. The PCA characterizing tissue from site I1 (Figure 7 a.) shows 4 genes loaded more strongly onto component 1, which described 46.5% of the variance, while 3 genes loaded more strongly onto component 2, which described 21.3% of the variance. PCA characterizing gill tissue from site I2 (Figure 7 b.) shows 6 genes loading more strongly onto component 1, which described 54.3% of the variance, which 1 gene loaded more strongly onto component 2, which described 20.1% of the variance. The PCA characterizing gill tissue from site I4 (Figure 7 c.) shows 6 of the genes loaded more strongly onto component 1, which described 49.2% of the variance, while 1 gene loaded more strongly onto component 2, which described 23.8% of the variance in the dataset. Figure 8 depicts PCA characterizing gill tissue from fall, spring, and summer. Figure 8 a. is a PCA

characterizing gill tissue from fall, 6 of the genes load more strongly onto component 1 which characterizes 56% of the variance, while the remaining gene loads more strongly onto component 2, which described 16.5% of the variance. PCA characterizing gill tissue from spring (Figure 8 b.) shows 4 of the genes loading more strongly onto component 1, which characterizes 42.9% of the variance, while 3 of the genes load more strongly onto component 2, which characterizes 21% of the variance. Tissue obtained from the summer is characterized by the PCA in Figure 8 c. and shows that 6 of the genes loaded more strongly onto component 1, which described 51.4% of the variance which 1 of the genes loaded more strongly onto component 2, which described 15.8% of the variance in the dataset.

4.5 *qPCR ANOVA gill tissue*

Expression values for the KCrec biomarker gene showed marginally significant differences between I2 and I4 in the fall (ANOVA, Tukey-Kramer, *P=.0714*), Figure 9. Expression values for the AS6 biomarker showed significant differences between the fall and summer seasons (ANOVA. Tukey-Kramer, *P=.0176*), Figure 10. Expression values for the EDL biomarker showed significant difference between control and impacted sites overall (ANOVA, Students-t test, *P=.0013*), Figure 14. EDL expression values were also significantly different and near significantly different between control and impacted sites in the spring, summer and fall seasons individually (ANOVA, Students-t test, *P=.0399*: *P=.0524: P=.0266*), Figures 11,12, and 13 respectively. There was also a significant difference found in expression values for EDL between sites C1 and I1 specifically overall (ANOVA, Tukey-Kramer, *P=.0010*), Figure 15. Expression values for the HSP70 biomarker were marginally different between control and impacted sites overall (ANOVA, Students-t Test, *P=.0859*), Figure 16. Expression values of the PRDX6 biomarker showed significant difference between control and impacted sites overall

(ANOVA, Students-t Test, *P=.0315*), Figure 22. There was also a difference in expression values of PRDX6 between control and impacted sites specifically in the summer season (ANOVA, Students-t Test, *P=.01189*), Figure 23. In a comparison between individual sites, a significant difference in expression values for PRDX6 was found between sites C1 and I1 specifically (ANOVA, Tukey-Kramer, *P=.0316*), Figure 20. In a comparison between all sites, there was a significant difference in expression values of PRDX6 between sites I2 and I4 in the fall specifically (ANOVA, Tukey-Kramer, *P=.0119*), Figure 21. Expression values of the GS biomarker were significantly different between control and impacted sites overall (ANOVA, Students-t Test, *P=.0286*), Figure 25. Expression values for the GS biomarker were also significantly different between control and impacted sites specifically in the spring (ANOVA, Students-t Test, *P=.0096*), Figure 24.

5. **DISCUSSION**

In the present study, the response of oyster genes in body and gill tissue from potentially impacted and control sites varying in temporal and spatial location was investigated. The majority of significant or nearly significant differences in biomarker gene expression observed were more frequently in the oyster gill tissue than in the mantle which indicates that gills are more sensitive to biotic and abiotic stressors (Meistertzheim, Tanguy, Moraga, & Thebault, 2007), as shown in other studies.

5.1 *Stress response genes in gill tissue*

Out of the 7 genes used as biomarkers, PRDX6 and HSP70 are the two that fall into the category of general stress response genes, i.e. not specifically related to immune stress response or metabolic stress response. In this study a significant difference was found in the expression of

PRDX6 in the gill tissue from control and potentially impacted sites over all seasons and specifically between the summer and fall seasons, there was an increase in gene expression for disturbed sites. As temperatures rise in the summer season oysters are subjected to a more stressful environment, it has been shown that elevated temperatures cause stress on oyster populations (Yang et al., 2017; H. Zhang et al., 2019). PRDX6 is a cellular stress response gene that acts as an antioxidant, an increase in oxidative stress arising from an increase in temperature or bacterial infection would cause an increase in the expression of this gene (Genard et al., 2013; Genard et al., 2012). While the fluctuating temperatures between season do not rely on native oysters proximity to an aquaculture site, it is important to note that elevated temperatures have been shown to cause changes in host susceptibility to pathogens making the oyster more likely to become infected by diseases like bacterial infections, parasites, and viruses (Wang et al., 2012; H. Zhang et al., 2019). If native oyster populations become more susceptible to disease when temperatures rise and those populations are in close proximity to cultured oysters, which are thought to be more susceptible to parasitic and bacterial infections due to over-crowding and stress from handling and husbandry (Kuchel et al., 2012) it could lead to a higher chance of invasive diseases traveling to and infecting the native reefs close by this could explain why an increased expression of the PRDX6 gene was seen in potentially impacted site, or those natural oyster reefs in close proximity to aquaculture sites. Infection from diseases like MSX, Dermo and ROD are known to cause mass mortality events in native oyster populations and their infections pose a severe threat to all oyster populations (Biancani et al., 2012; Forrest et al., 2009; Piontkivska et al., 2011). PRDX6 also showed a significant change in expression specifically between sites C1 and I1 in all seasons and between sites I4 and I2 in the fall seasons. Site I4 is located by an aquaculture farm that was more active and used floating bags, while site

I2 is located by a farm that was less active and used bottom bags. Site I4 would have been subjected to an increase in mechanical disturbances for husbandry since the farm that it is next to was tended to often, oysters have been shown to exhibit stress responses upon mechanical agitation and this type of disturbance can cause an increase in the expression of general stress related genes (Lacoste et al., 2001; Lacoste et al., 2002). The cause of difference in expression between sites C1 and I1 in particular are not completely clear. As previously mentioned there are several biotic and abiotic factors that could cause a change in the expression of an antioxidant gene like PRDX6, this specific difference may be an anomaly, as discussed in the conclusion it is difficult to elucidate minute specific differences due to the unexpected reducing of sample size for this study.

HSP70, the other stress response gene analyzed showed marginally significant differences found in HSP70 gene expression for gill tissues in control and potentially impacted sites over all seasons. HSP70 is a chaperone protein and responds to the same stressors that the PRDX6 genes does, due to this it is expected that if there were an increase in expression of PRDX6 there would also be an increase in the expression of HSP70. However, it is notable that while PRDX6 showed significant differences between control and potentially impacted sites, HSP70 did not. Because PRDX6 and HSP70 are both stress response related genes, we would expect them to cluster together on PCA, however they did not for the majority of PCAs, indicating that they are not acting similarly or are responding to different stimuli. PRDX6 is typically either on its own or clustered with a single other gene, which differs depending on the variables, on component 2 of each PCA while HSP70 tends to cluster with the majority of other genes on component 1. The tendency of PRDX6 to cluster on its own could indicate that the

activity and expression of PRDX6 in *Crassostrea virginica* is not consistent over a variety of variables and may not be a good candidate as a biomarker for future studies.

5.2 *Metabolic response genes in gill tissue*

ATP synthase f0 subunit 6 (AS6), endothelial lipase (EDL), and glutamine synthetase (GS) were the three metabolic stress response genes analyzed in this study. Gene expression differences in AS6 were observed between the summer and fall seasons over all samples. AS6 gene codes for a protein that is part of the electron transport chain so is related to energy metabolism in cells, it has been shown that an increase in temperature causes metabolic stress in oysters leading to a decrease in the expression of this gene (Genard et al., 2012; Ivanina et al., 2013; Piontkivska et al., 2011), the change in temperature between the summer and fall could explain the differences in gene expression levels between these two seasons. Metabolic regulation, including metabolic rate depression, allows bivalves to survive prolonged periods of extreme stress related to drastic fluctuations in salinity, pH, oxygen and carbon dioxide concentrations in the intertidal zone (Altieri, 2006; Gracey et al., 2008; Guppy, Fuery, & Flanigan, 1994; Ivanina & Sokolova, 2013), so while metabolic control via rate depression would lead to a decrease in the expression of AS6 this may not be related to stress due to aquaculture proximity but may be a natural response to typical stressors oysters experience particularly since this difference in expression was seen between seasons and not between control and potentially impacted sites specifically. As see in Figure 8 c. AS6 and PRDX6 grouped together on component 1 in the PCA conducted on genes and seasons which could indicate that their expression is related in some way or is part of the same pathway. Based on analysis in this study it seems that PRDX6 and AS6 are inversely related, while an increase in temperature brings about metabolic stress leading to a decrease in the expression of AS6 it

causes an increase in the expression of PRDX6 which is expected based on the functions of these genes and what their expected change in expression is based on previous studies.

In the current study differences in gene expression of EDL were found between control and potentially impacted sites in all seasons. EDL gene expression differences were also seen particularly between sites I1 and C1. EDL is similar to AS6 in that it is a gene that participates in an oysters metabolic pathway and we would expect to see a decrease in its expression under conditions of metabolic stress such as hypoxia, or an increase in temperature or salinity (Genard et al., 2012; Ivanina et al., 2013). By definition, the introduction of oysters for the purpose of aquaculture increases the number of oysters in a given area, with an increase in oyster population density there is also an increase in rates of oyster bio deposition, and this can increase sedimentation rates. (Grizzle et al., 2008; Higgins et al., 2013; Hoellein et al., 2015) Increased sedimentation can potentially decrease sediment and water column oxygen concentrations (Dahlback & Gunnarsson, 1981; Kaiser et al., 1998), these hypoxic conditions could be the cause of EDL down regulation observed in oysters obtained from potentially impacted sites. The specific difference observed in EDL gene expression between sites I1 and C1 are unexpected. Because sediment burial can be accelerated by mesh, bottom bags (Comeau, 2014) it would be expected that the greater risk for hypoxia induced stress would be with those oysters from reefs closer to lease 2 (sites I2 and I3), when in fact it was site I1 that saw the most change in gene expression and it is located closest to lease 1 which has floating bags. It is possible that other variables for which we did not account caused this difference.

Changes in expression levels of the GS gene were observed between control and potentially impacted sites overall in the current study. The GS gene is part of the metabolic stress group and it behaved similarly to EDL, this is expected since its change in expression is due to

the same stressors as that gene e.g. elevated temperatures and salinity as well as hypoxia. As previously stated, the difference between gene expression in control and potentially impacted sites could be attributed to hypoxic conditions from increased oyster density due to aquaculture sites. AS6, EDL and GS clustered together on component 1 of almost all PCAs, this indicates that the change in expression of these gene is closely related, these findings were expected due to the fact that these genes all participate in the metabolic pathway and all were expected to be down regulated within oysters exposed to common stressors amongst the three genes.

5.3 *Immune response genes in gill tissue*

While there were two genes from the immune response category used as biomarkers in this study (KCrec and SUP) only KCrec showed a difference in gene expression levels in gill tissue. KCrec had borderline significant differences in gene expression levels between two of the potentially impacted sites, I4 and I2. Site I2 was located closer to the mouth of the bay so may have been subjected to disturbances from human water activities such as kayaking, collecting of crab pots, and boat wakes, while site I4 was nestled farther up in the bay and was protected from those disturbances. While these particular results do not support the theory that aquaculture sites cause increased levels of stress on native oyster populations overall, they do indicate that those reefs exposed to more human contact and handling could be more susceptible to disease. With the addition of aquaculture comes more human involvement and activity, causing native reefs to be exposed to more disturbances (Forrest & Creese, 2006; Forrest et al., 2009), i.e. dispersal and maintenance of gear, vessel movements such as propeller wash, as well as harvesting of large amounts of oysters several times a year, (De Grave et al., 1998; Forrest & Creese, 2006). Bacterial infection and heat stress are two major environmental threats for oyster populations and elevated temperatures, while causing stress on their own, have been shown to cause changes in

host susceptibility to pathogens making the oyster more likely to become infected by disease (Yang et al., 2017; G. F. Zhang et al., 2012; H. Zhang et al., 2019) because of this relationship we would expect to see immune response genes and general stress response genes like HSP70 clustered together on PCA, which is what was observed in this study. Typically, HSP70 along with KCrec and SUP were shown to clustered together on component 1 of PCA indicating that their patterns of expression are similar.

5.4 *Gene responses in body tissue*

The only genes out of the 7 selected biomarkers that had a significant or nearly significant change in expression in the body tissue were PRDX6 and SUP, part of the general stress response and immune response gene categories respectfully. No genes from the metabolic stress gene group (AS6, EDL, GS) or the 2 other genes comprising the aforementioned groups (HSP70, KCrec) showed any significant differences in expression in oyster body tissue.

PRDX6 showed significant differences in expression between control and potentially impacted sites in the fall, borderline significant differences in the spring and significant differences between the summer and fall seasons overall. The differences in gene expression levels of PRDX6 in the body tissue may indicate that there was a particularly high level of pest and/or disease present in oysters from the potentially impacted sites or that there was a particularly drastic increase in temperature between the summer and fall seasons. Because oysters are filter feeders the gene expression of their gills is much more susceptible to changes in the environment (Meistertzheim et al., 2007) so for the body tissue of the oyster to respond to a stressors it may have been particularly sever stressor(s). The fact that the differences seen in expression were seasonal could indicate that the initial stressor was temperature fluctuation

which is shown to make oysters more susceptible to other stressors like disease or pests, causing a change in the expression of PRDX6.

The only significant or borderline significant change in expression for the SUP gene observed in this study was the difference in expression between I2 and all other sites. We would expect to see an increase in the expression of SUP if the oysters were exposed to pesticides or pollutants since SUP is a known biomarker of genotoxic damage (Ciocan & Rotchell, 2005; Lima et al., 2008). The reason that site I2 differs in expression of SUP from all other sites sampled is not clear, while there was a difference between control and potentially impacted sites there was also a difference between I2 and all other potentially impacted sites. Due to tidal changes and water flow in the bay where all sites were located, it seems unlikely that only one of the sites would be subjected to this type of chemical exposure while none of the others were.

Although there were no significant differences seen in oyster body tissue for the GS gene, there were a few instances upon PCA where it was observed that GS clustered on component 2 when AS6 and EDL were clustered on component 1, this was observed only in oyster body tissue. This difference in clustering could mean that expression level changes in GS were, at times, behaving differently than expected. It has been shown that an increase in the expression of GS can occur upon xenobiotic exposure (Tanguy, Boutet, & Moraga, 2005; Tate, Meister, & Leu, 1972), while there were no significant differences found in expression of GS in any body tissue this could be a possible explanation for the differences seen between these three genes in PCA.

6. **CONCLUSIONS**

This study provides further information on the possible effects of oyster aquaculture along with seasonal and spatial differences on native populations of Eastern oyster *Crassostrea virginica.* This research shows that there is a difference in gene expression between naturally occurring reefs that are closer to aquaculture sites and naturally occurring reefs that are farther from aquaculture sites. It is important that in further studies related to possible effects of aquaculture on native oyster populations or in studies related to effects of gene expression due to stress in oysters that a larger sample size be used for analysis. Having a larger sample size could have helped the researches elucidate patterns of gene expression over time and space more clearly and would have given the researcher more power when performing data analysis. Although some trends such as, higher changes in gene expression of gill tissue, higher changes of gene expression during the summer and fall seasons, and were seen further research in this area using a larger sample size coupled with a wider array of biomarkers could help these patterns become more clear. It is also important to note that none of the genes used in this study change expression levels based only on a single stressor, typically there are a host of stressors that are able to cause a change in expression of a single gene. Further research in this area, using a larger sample size, may help to clarify the effect of some of the confounding variables presented in this study. While this study did show that there are clearly differences in stress related gene expression levels between native oyster populations based on their proximity to aquaculture sites, more research is needed to further understand the ultimate consequences, if any, this increase in stress is having in the native populations.

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Table 1. Target gene data. Characteristics of primers and efficiency percentages (E%) of genes used as biomarkers and reference genes.

Gene	Symbol	Function	E%	Forward Primer Sequence	Reverse Primer Sequence
Killer cell lectin-like receptor	KC-rec	Non-self recognition (lectin receptor family)	99	GGTCTTTGCCAGTTTCGGGTTTATAAC	TGGTCTGCGGAGACACCAATARGGCCT
ATP synthase f0 subunit 6	AS6	Energy metabolism (complex V of electron transport chain)	103	ATGCCAAGCATGTTCTACAGAGT	GCAAAGGATCGCTCCTACCAAAGC
Endothelial lipase precursor	EDL	Lipid metabolism (phospholipase)	95	GCCCACACCATGGGATACGCCGG	TGGCACCCCGGTTGTCTCGTCCC
Heat shock protein 70	HSP70	Chaperone protein (cytoprotection)	102	ATGAGTAAACACCAACAGGCCATCGG	AAGATAGTGTTCGTAGGGTTCATGG
Peroxiredoxin 6	PRDX6	Oxidative stress (antioxodant enzyme)	101	GATGACGTCCCCAGTCATGAGGGGTGGTC	TGGGGGATGGAGGGTAAGACCATACACTT
Glutamine synthetase	GS	Protein metabolism (amino acid synthesis)	98	ACGGAGGTTGACGGGACTT	GCTGGCACCACGATTGG
RAS supressor	SUP	Cell division inhibitor	98.5	ACTGAAGTTGTGGAGGCTAAGGCT	ATTCCTAGATTTTTCTCCACACC
Ribosomal protein 18s	R18s	Reference	95	GTCTGGTTAATTCCGATAACGAACGGAACTCTA	TGCTCAATCTCGTGTGGCTAAACGCAACTTG
Beta-actin	BActin	Reference	95	TTGGACTTCGAGCAGGAGATGGC	ACATGGCCTCTGGGCACCTGA

Table 2. Summary of all principal components analysis (PCA) data

Comparison	Tissue	Component 1 Percent and Number of Genes	Component 2 Percent and Number of Genes
control sites	body	50.6% : 6 genes	22.5% : 1 gene
possible impacted sites	body	32.4% : 4 genes	21.5% : 3 genes
control site 1	body	42.4% : 3 genes	27.1% : 4 genes
control site 2	body	49.9% : 6 genes	23.5% : 1 gene
control site 6	body	56% : 5 genes	26.4% : 2 genes
possible impacted site 1	body	45.3% : 4 genes	29.1% : 3 genes
possible impacted site 2	body	42.4% : 4 genes	25.2% : 3 genes
possible impacted site 4	body	46.8% : 5 genes	29.1% : 2 genes
fall	body	47.7% : 6 genes	19.2% : 1 gene
spring	body	33.3% : 4 genes	28.4% : 3 genes
summer	body	50.3% : 6 genes	21.4% : 1 gene
control sites	gill	55.3% : 6 genes	15.6% : 1 gene
possible impacted sites	gill	45.2% : 7 genes	16.1% : 0 genes
control site 1	gill	55.8% : 5 genes	19.2% : 2 genes
control site 2	gill	40.3% : 4 genes	29.6% : 3 genes
control site 6	gill	73.6% : 7 genes	16.6% : 0 genes
possible impacted site 1	gill	46.5% : 4 genes	21.3% : 3 genes
possible impacted site 2	gill	54.3% : 6 genes	20.1% : 1 gene
possible impacted site 3	gill	49.2% : 6 genes	23.8% : 1 gene
fall	gill	56% : 6 genes	16.5% : 1 gene
spring	gill	42.9% : 4 genes	21% : 3 genes
summer	gill	51.4% : 6 genes	15.8% : 1 gene

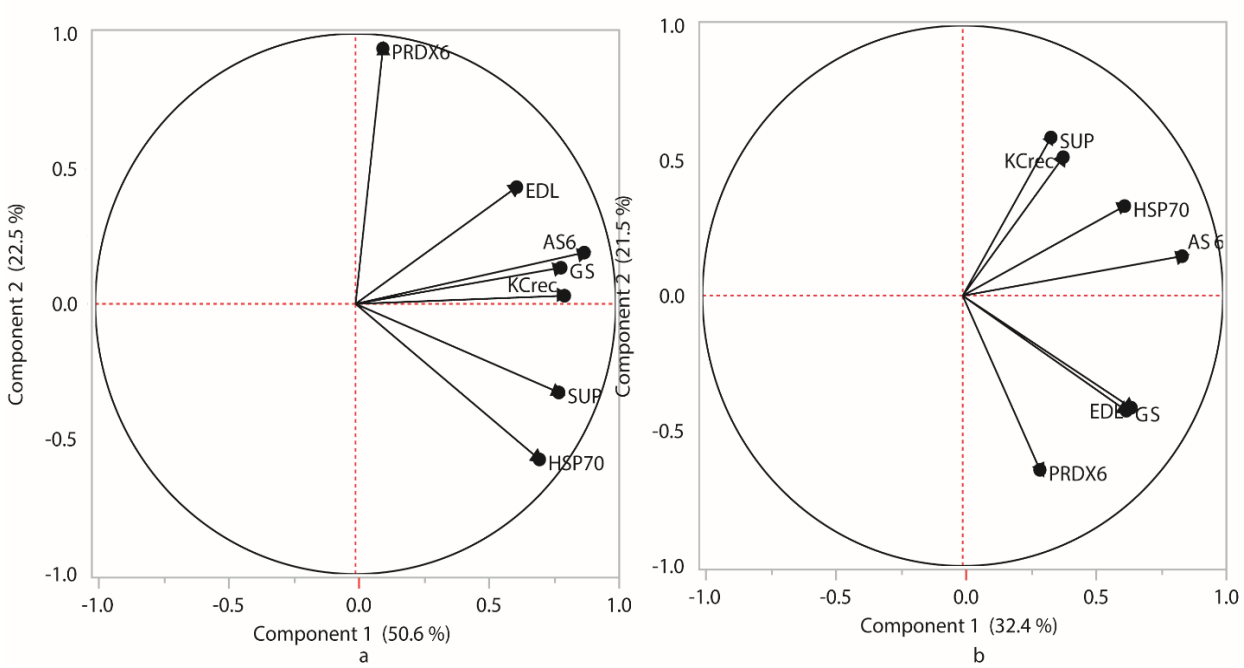


Figure 1. Principal components analysis biplots showing MEVs (mean expression values) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* body tissue from a. control sites and b. possible impacted sites. The direction of the arrows represents alignment with component 1(50.6% and 32.4%) or component 2 (22.5% and 21.5%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

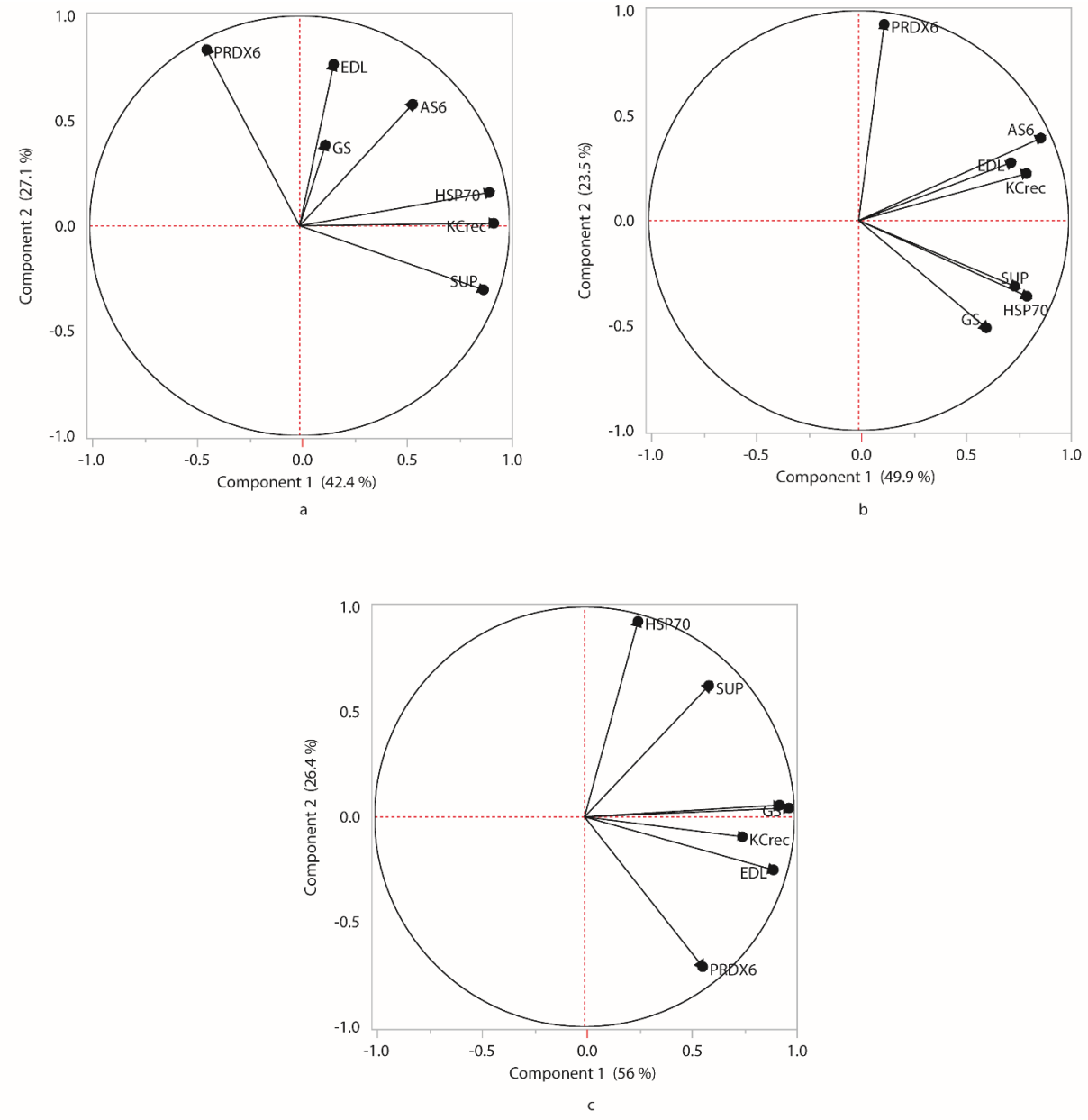


Figure 2. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* body tissue from a. control site 1 (C1) b. control site 2 (C2) c. control site 6 (C6). The direction of the arrows represents alignment with component 1(42.4%, 49.9%, and 56%) or component 2 (27.1%, 23.5%, and 26.4%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

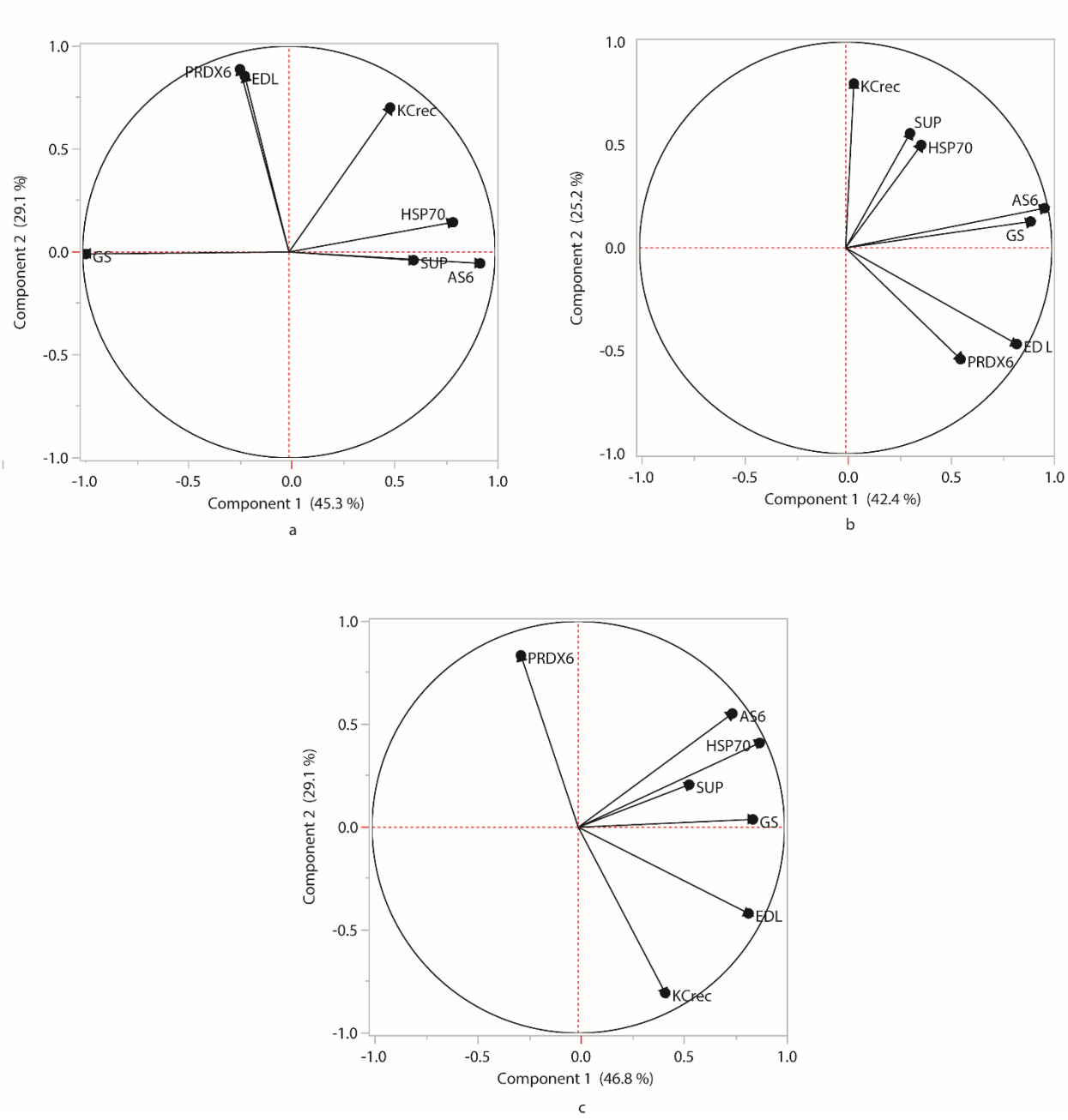


Figure 3. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* body tissue from a. impacted site 1 (I1) b. impacted site 2 (I2) c. impacted site 4 (I4). The direction of the arrows represents alignment with component 1(45.3%, 42.4%, and 46.8%) or component 2 (29.1%, 25.2%, and 29.1%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

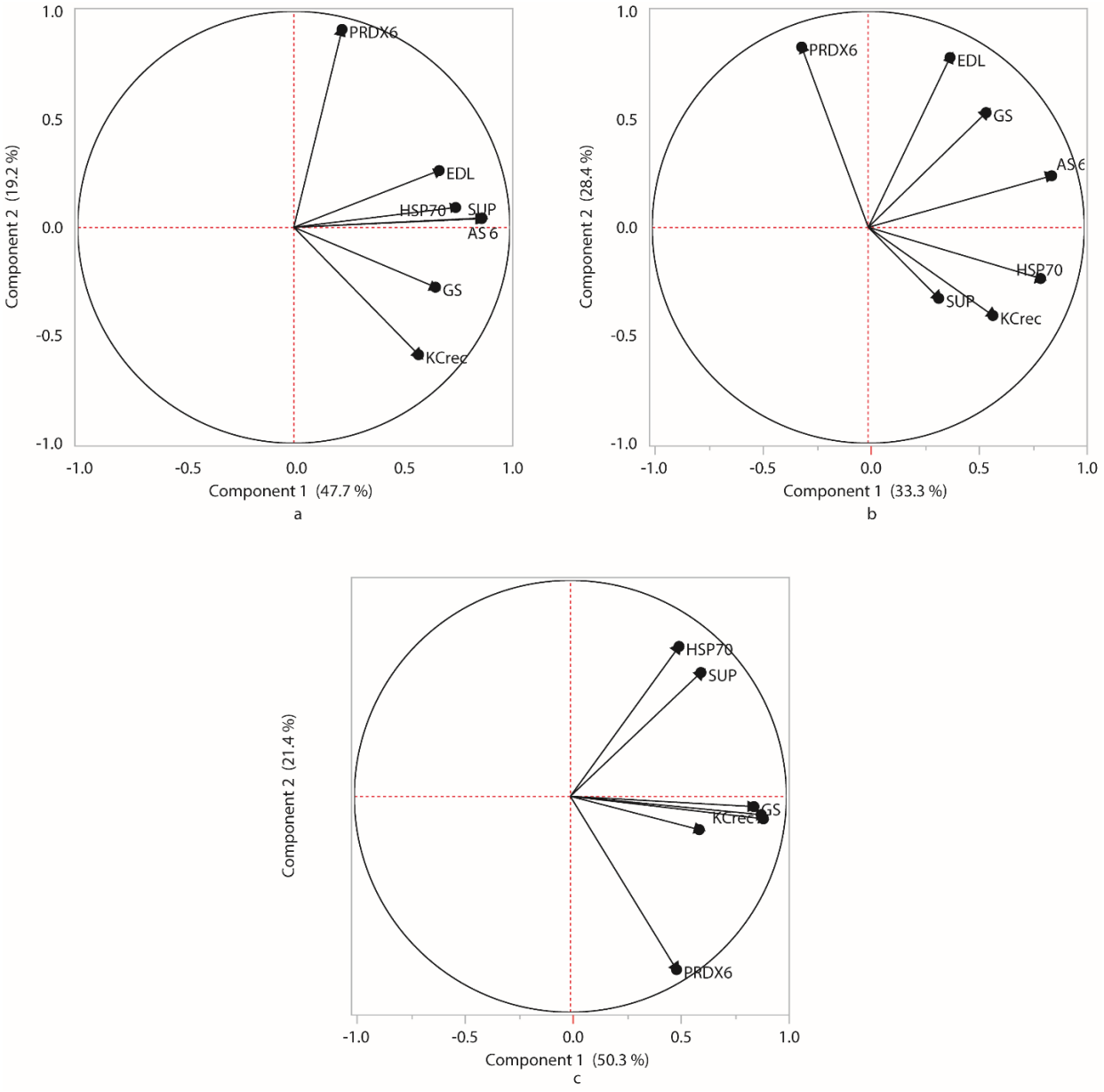


Figure 4. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* body tissue from a. fall b. spring c. summer. The direction of the arrows represents alignment with component 1(47.7%, 33.3%, and 50.3%) or component 2 (19.2%, 28.4%, and 21.4%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

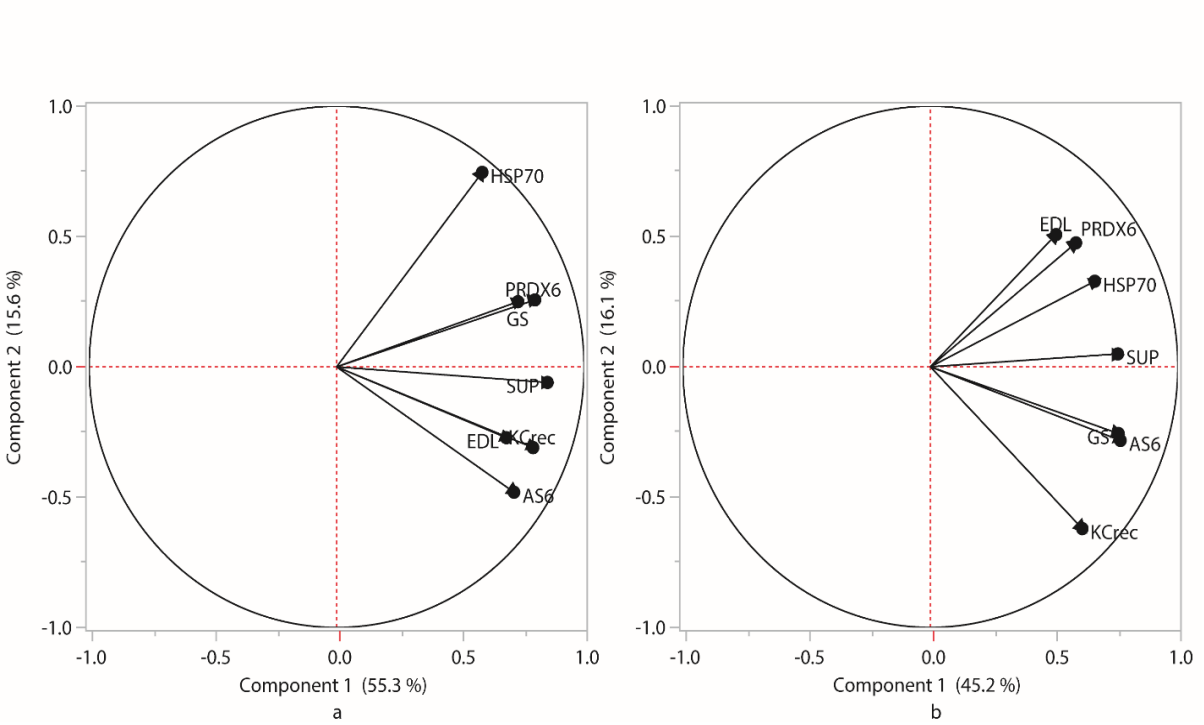


Figure 5. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* gill tissue from a. Control sites b. Possible impacted sites. The direction of the arrows represents alignment with component 1(55.3% and 45.2%) or component 2 (15.6% and 16.1%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

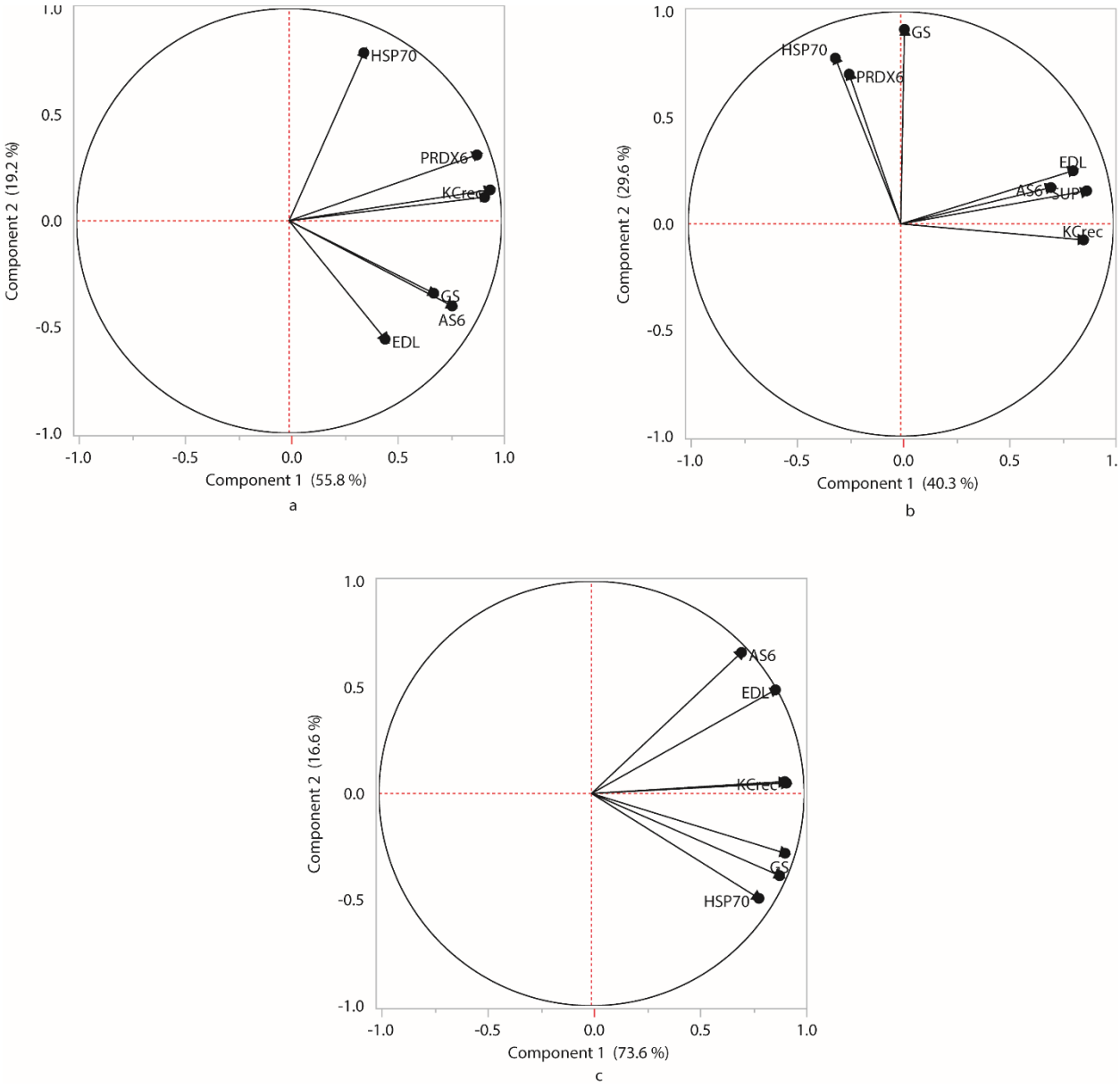


Figure 6. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* gill tissue from a. Control site 1 (C1) b. Control site 2 (C2) c. Control site 6 (C6). The direction of the arrows represents alignment with component 1(55.8%, 40.3%, and 73.6%) or component 2 (19.2%, 29.6%, and 16.6%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

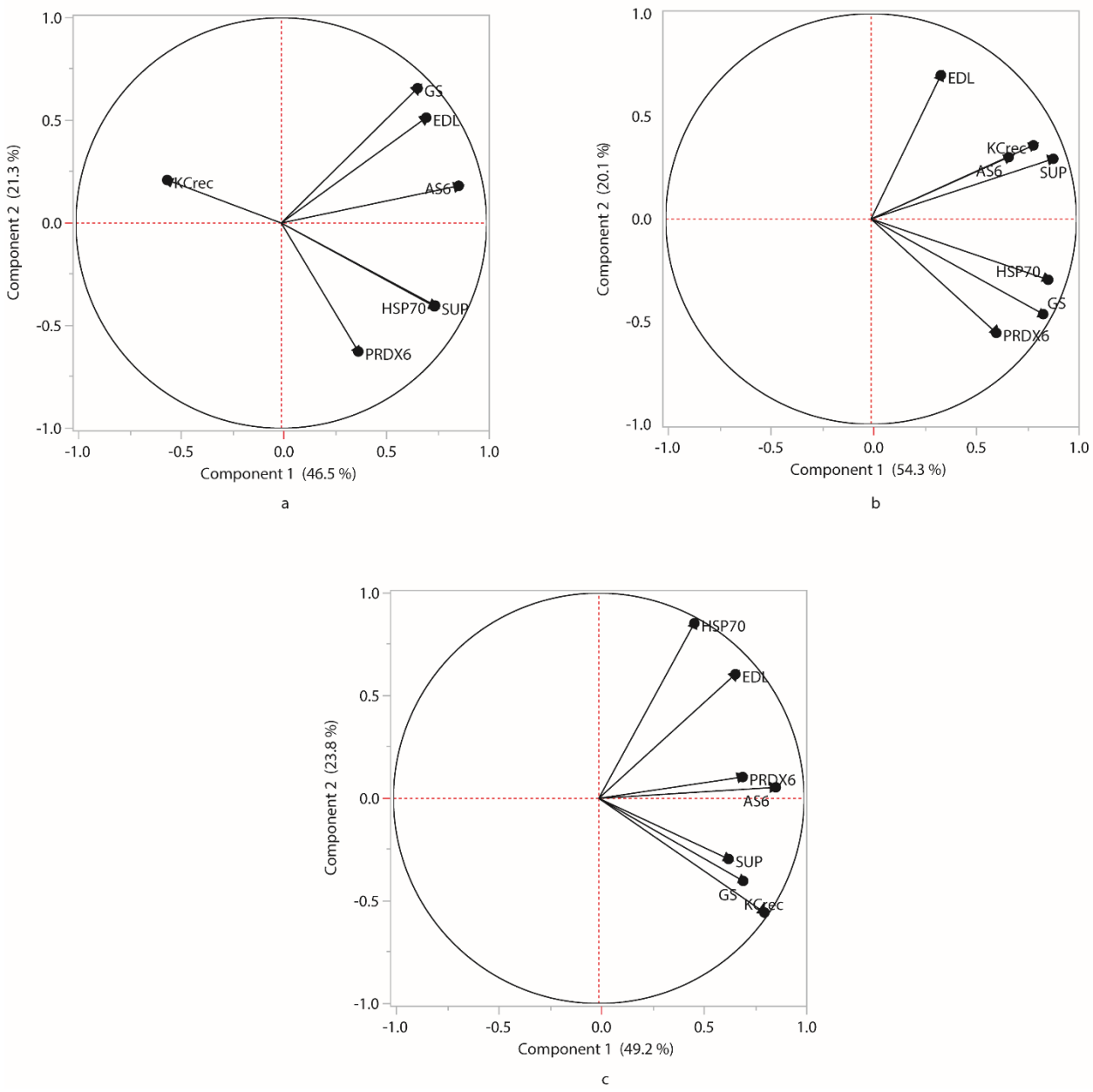


Figure 7. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* gill tissue from a. Impacted site 1 (I1) b. Impacted site 2 (I2) c. Impacted site 4 (I4). The direction of the arrows represents alignment with component 1(46.5%, 54.3%, and 49.2%) or component 2 (21.3%, 20.1%, and 23.8%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

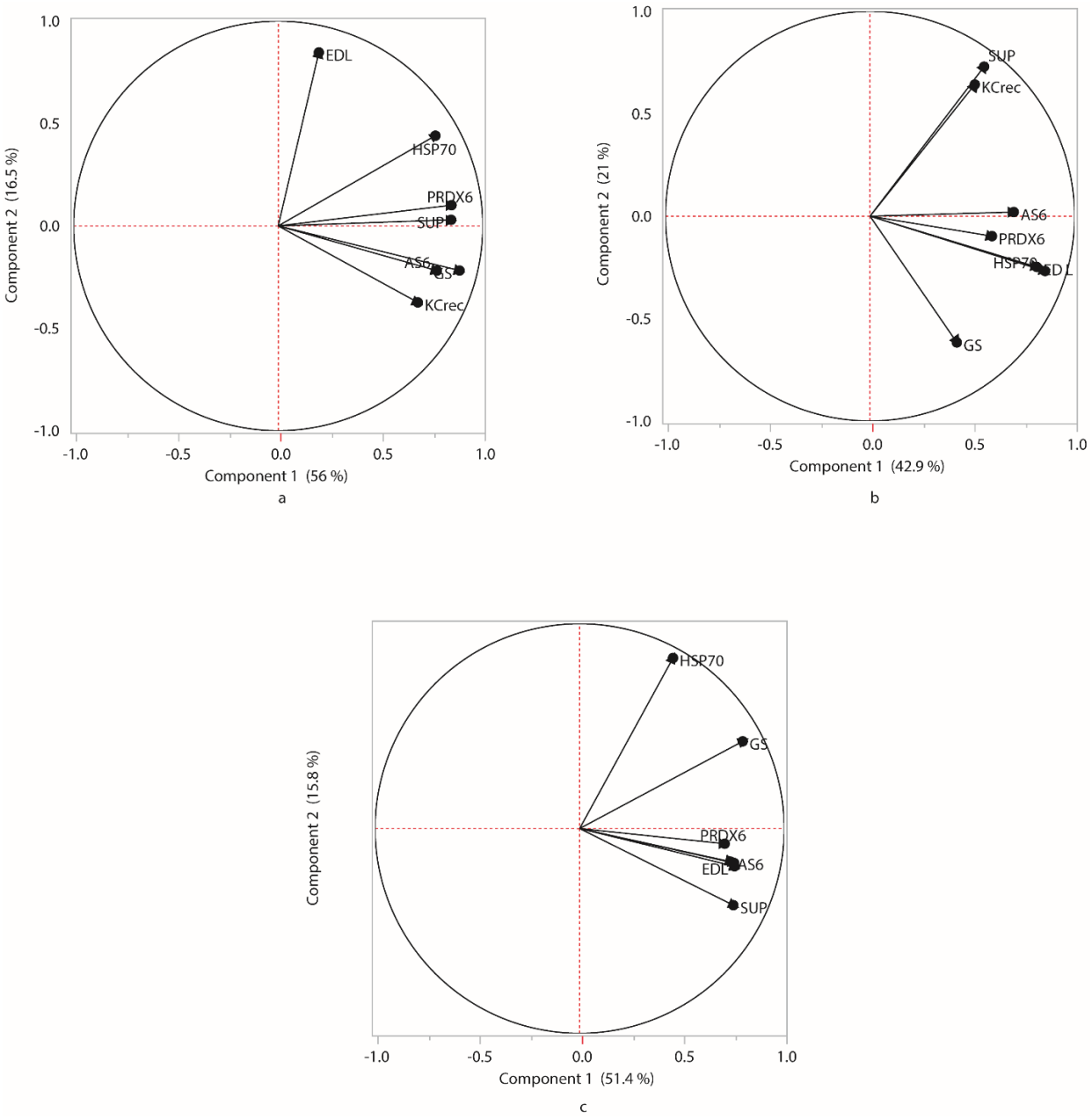


Figure 8. Principal components analysis biplots showing mean expression values (MEVs) from seven biomarker genes analyzed via qPCR in *Crassostrea virginica* gill tissue from a. fall b. spring c. summer. The direction of the arrows represents alignment with component 1(56%, 42.9%, and 51.4%) or component 2 (16.5%, 21%, and 15.8%). Arrows pointing in the same direction, aligning with one component, are more similar to one another.

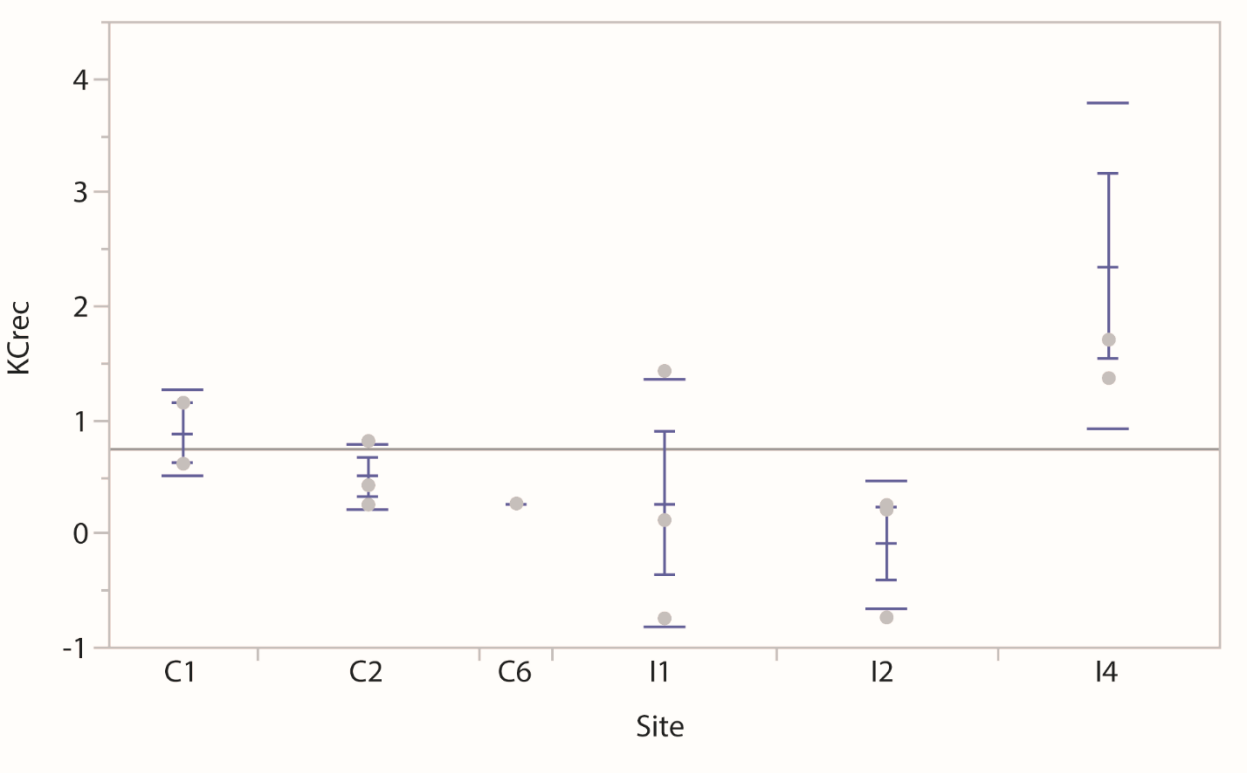


Figure 9. One way analysis of variance (ANOVA) of the KCrec biomarker in gill tissue of *Crassostrea virginica* between all sites from the fall season (September to October 2016).

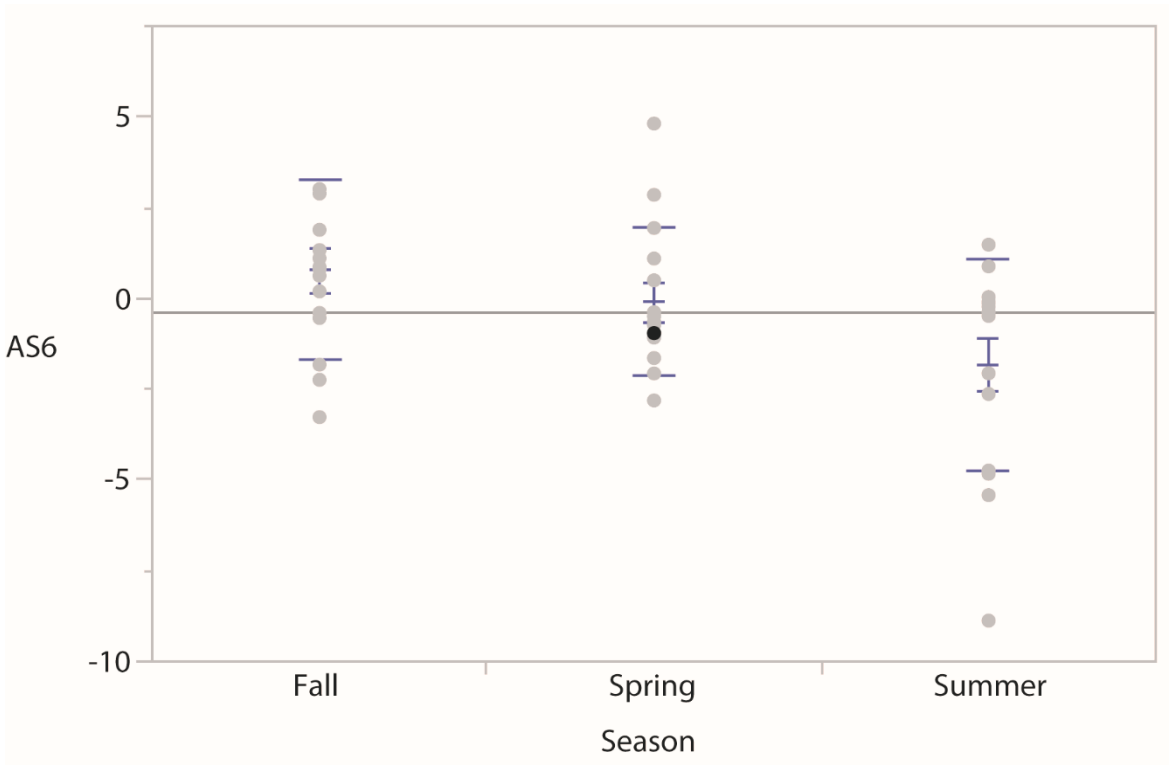


Figure 10. One way analysis of variance (ANOVA) of the AS6 biomarker in gill tissue of *Crassostrea virginica* between three seasons (fall, spring, and summer)

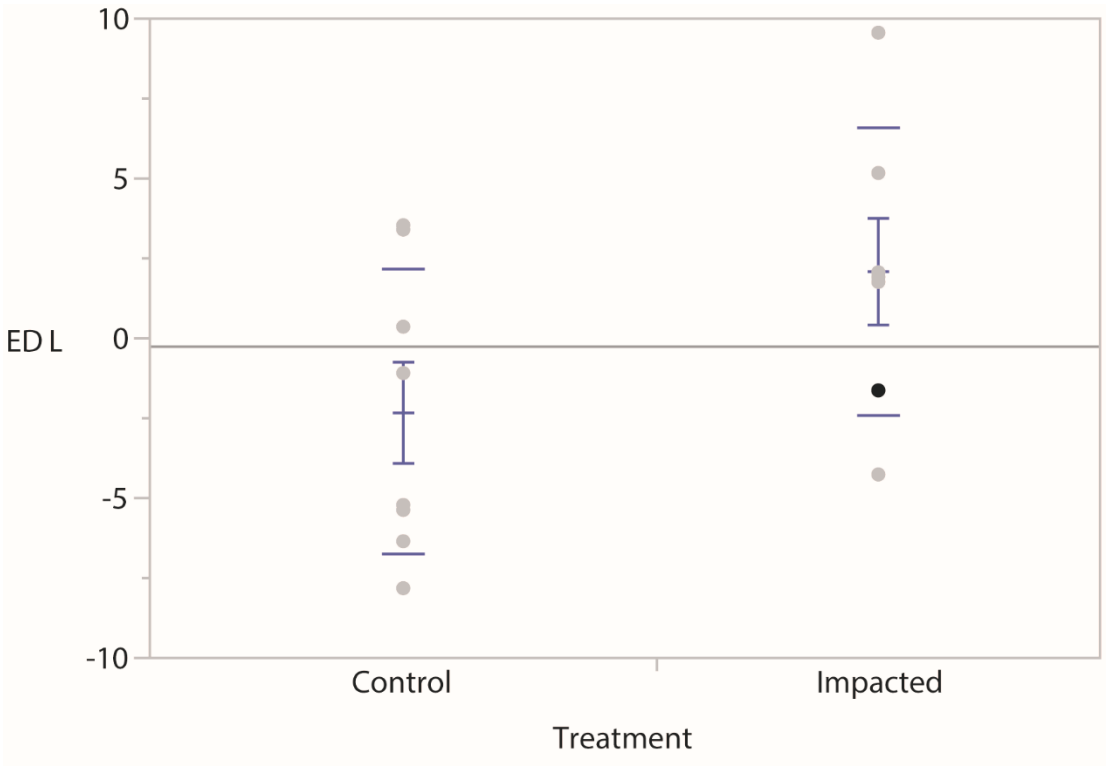


Figure 11. One way analysis of variance (ANOVA) of the EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017)

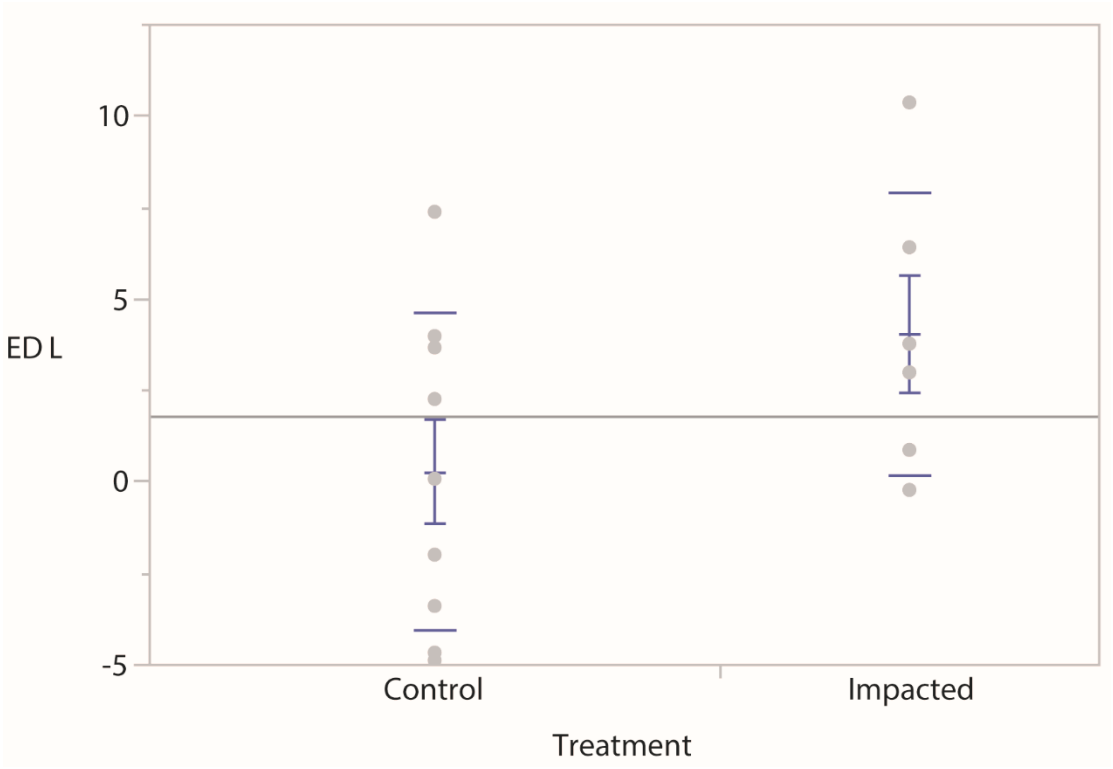


Figure 12. One way analysis of variance (ANOVA) of the EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the summer season (May to June 2017).

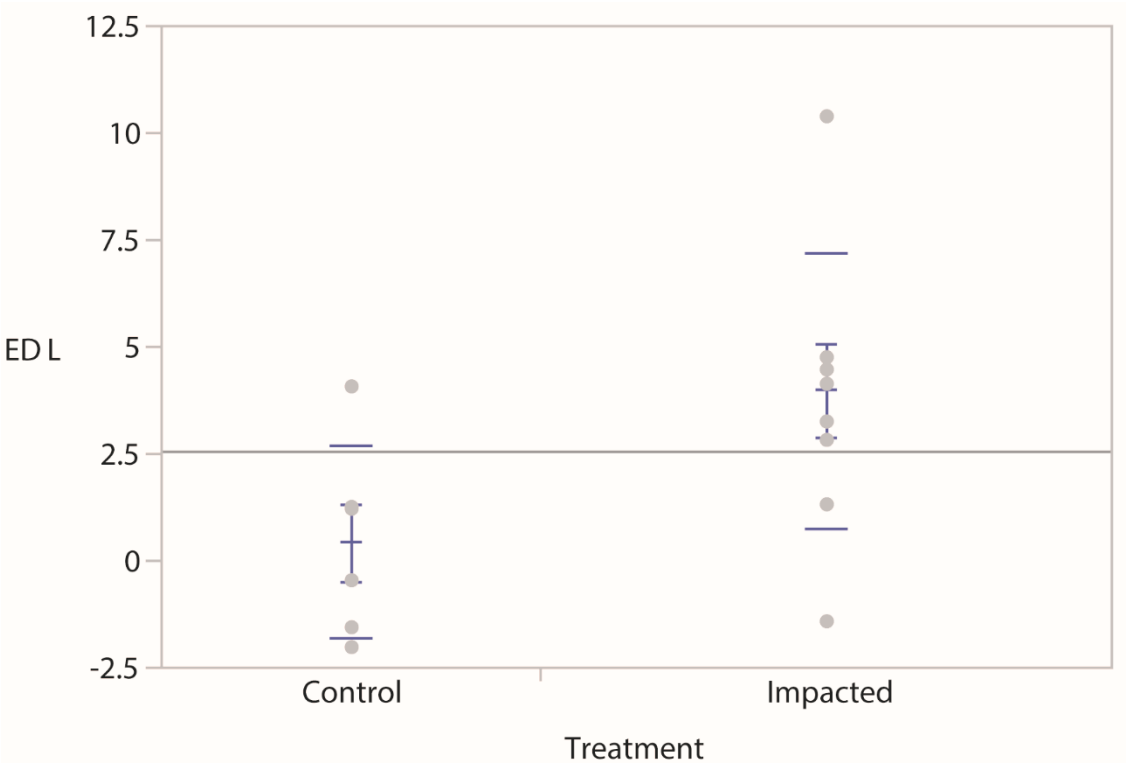


Figure 13. One way analysis of variance (ANOVA) of the EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the fall season (September to October 2016).

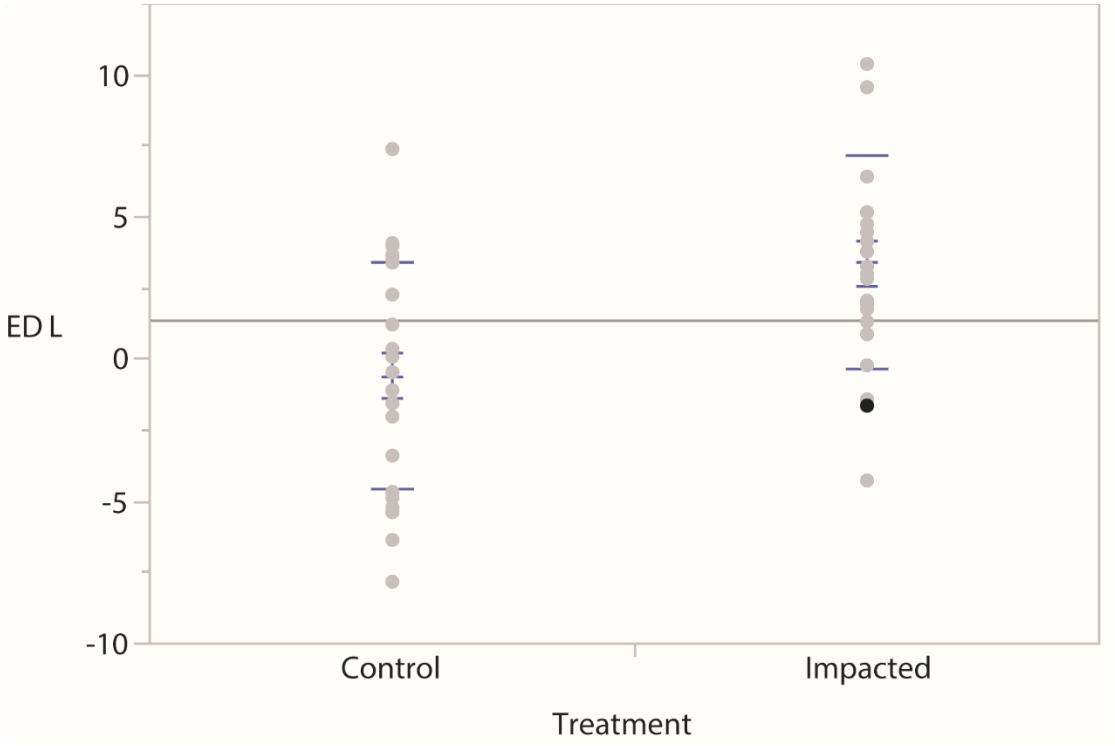


Figure 14. One way analysis of variance (ANOVA) of the EDL biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

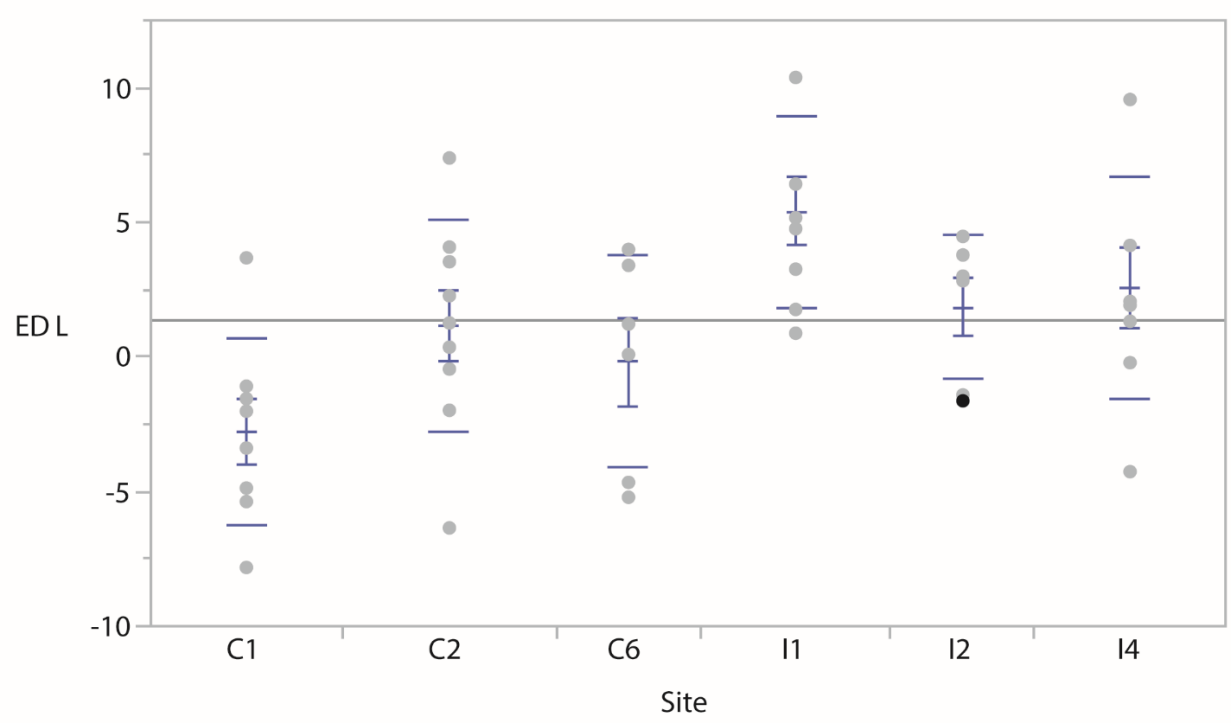


Figure 15. One way analysis of variance (ANOVA) of the EDL biomarker in gill tissue of *Crassostrea virginica* between all sites.

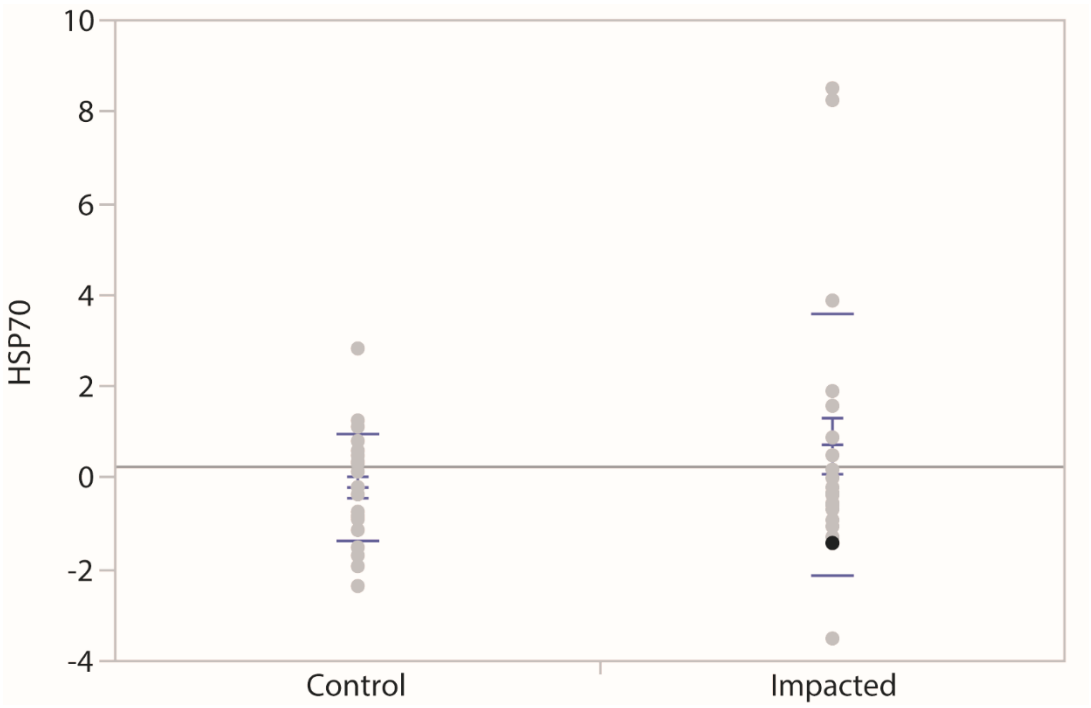


Figure 16. One way analysis of variance (ANOVA) of the HSP70 biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

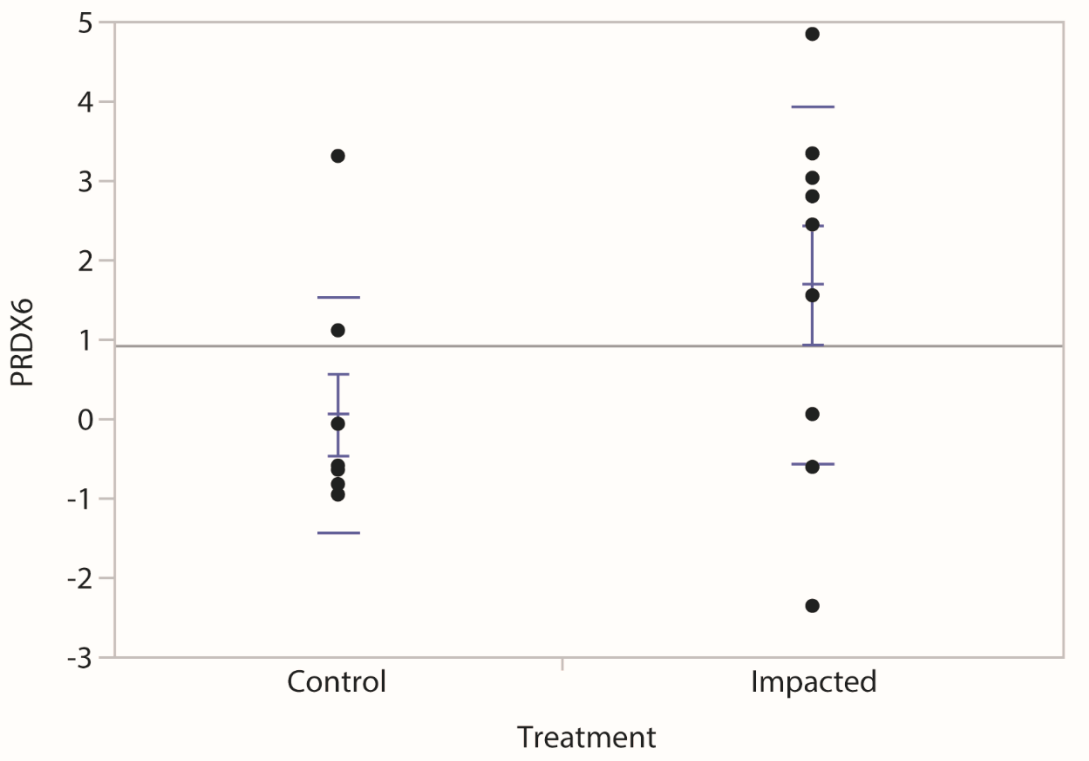


Figure 17. One way analysis of variance (ANOVA) of the PRDX6 biomarker in body tissue of *Crassostrea virginica* between control and impacted sites from the fall season (September to October 2016).

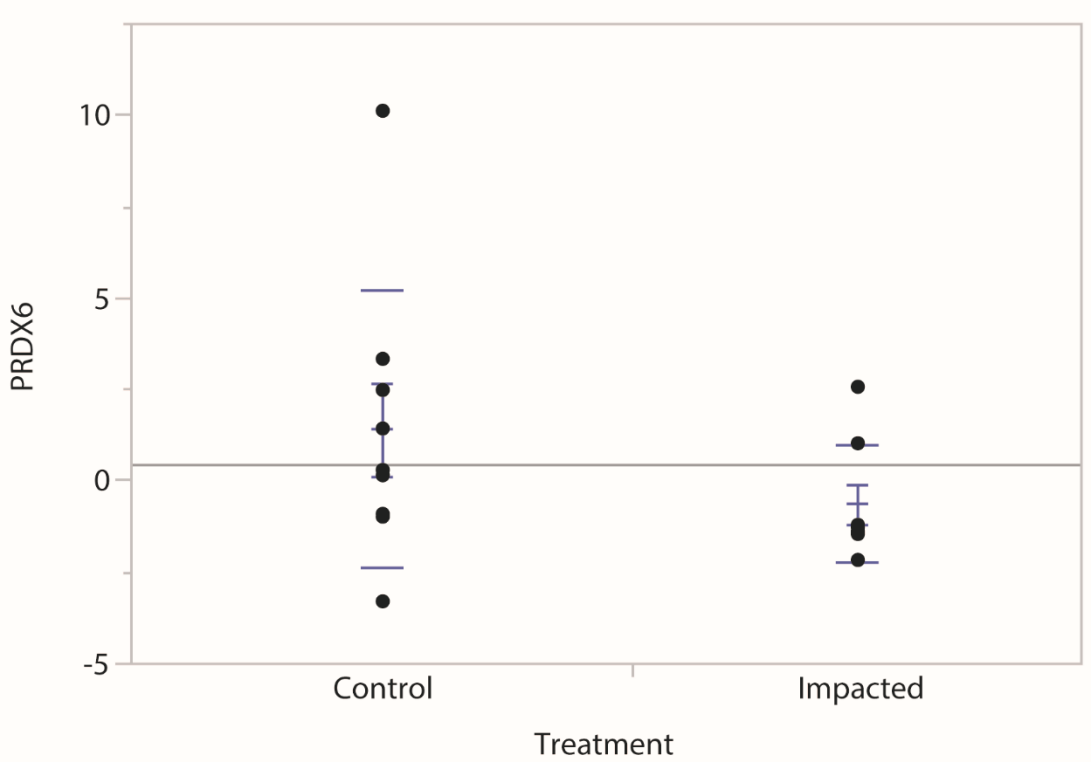


Figure 18. One way analysis of variance (ANOVA) of the PRDX6 biomarker in body tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017).

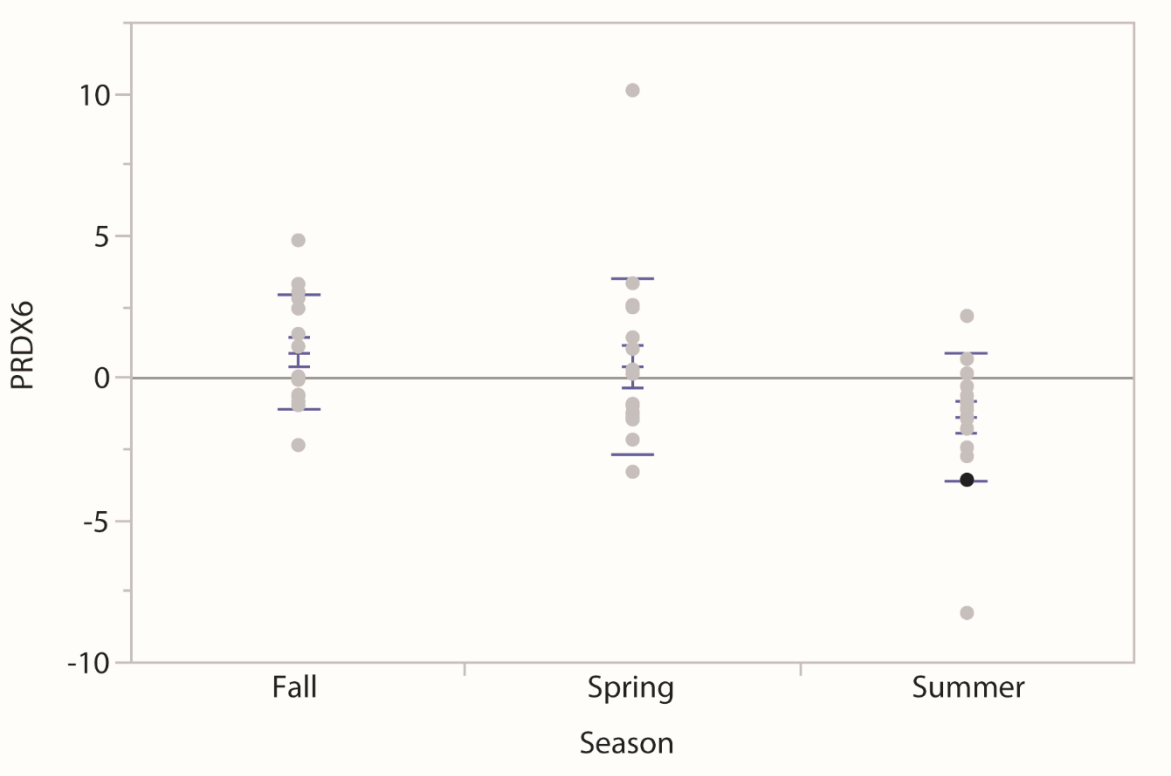


Figure 19. One way analysis of variance (ANOVA) of the PRDX6 biomarker in body tissue of *Crassostrea virginica* between three seasons (fall, spring, and summer).

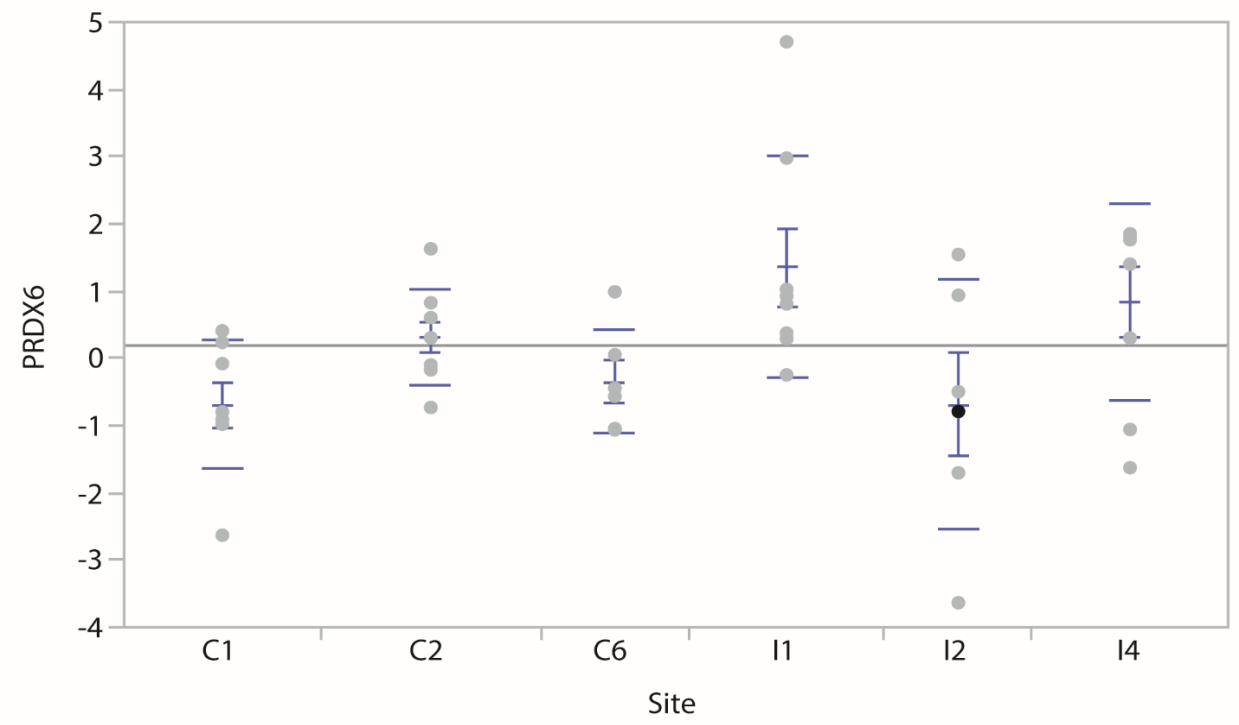


Figure 20. One way analysis of variance (ANOVA) of the PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all sites.

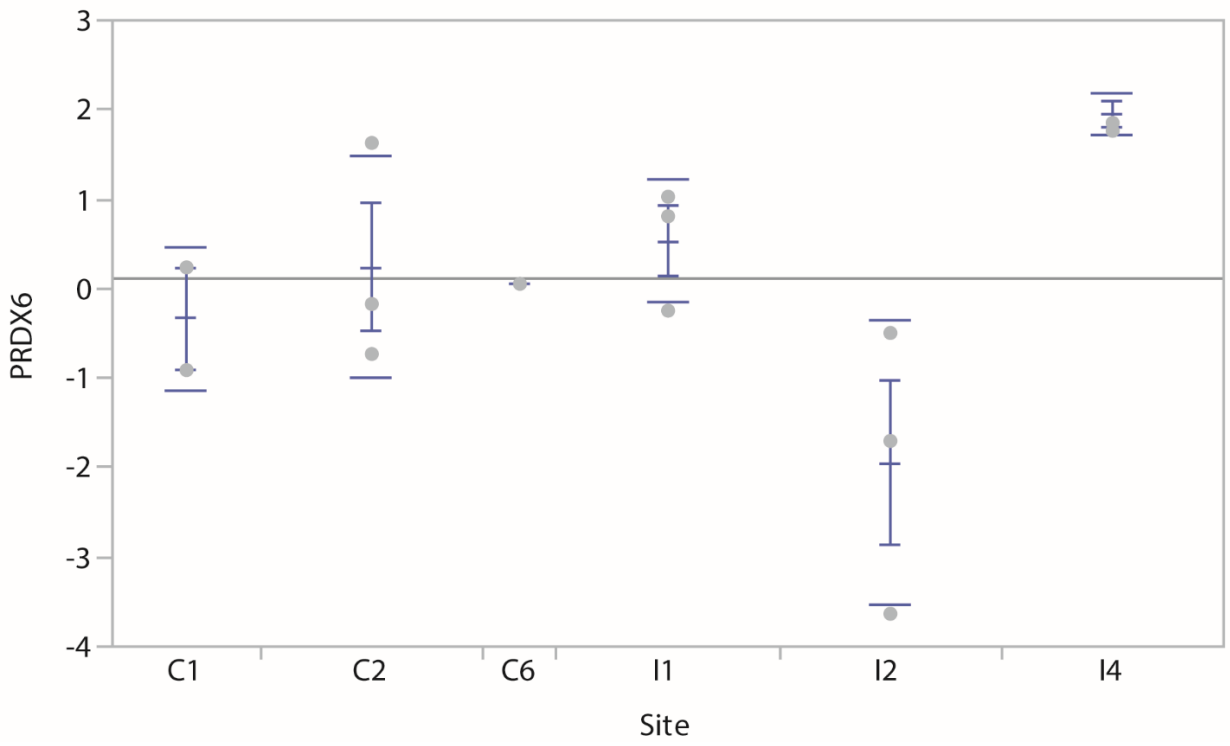


Figure 21. One way analysis of variance (ANOVA) of the PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all sites within the fall season (September to October 2016).

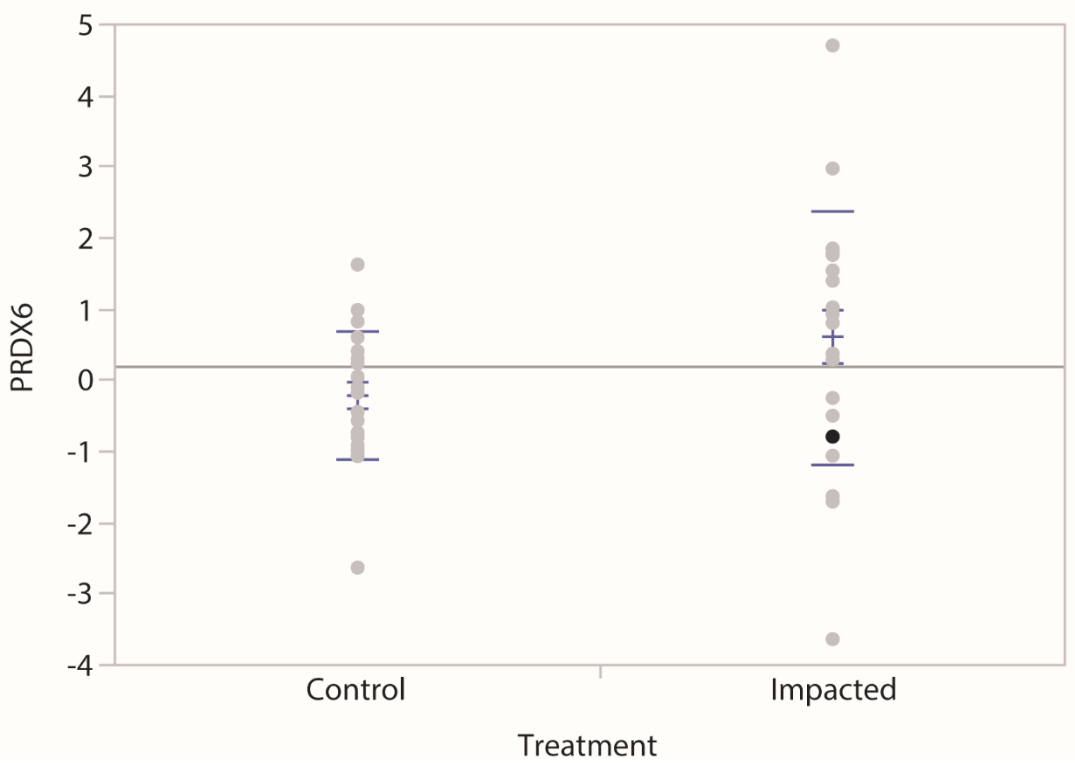


Figure 22. One way analysis of variance (ANOVA) of the PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

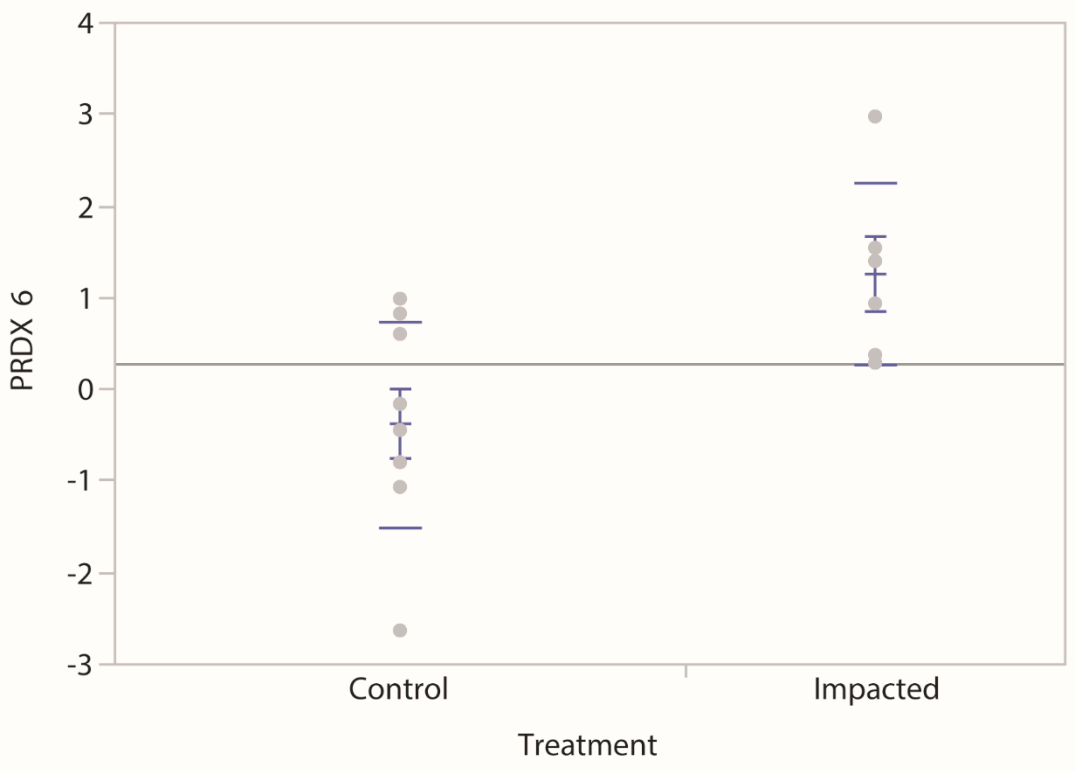


Figure 23. One way analysis of variance (ANOVA) of the PRDX6 biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the summer season (June 2017).

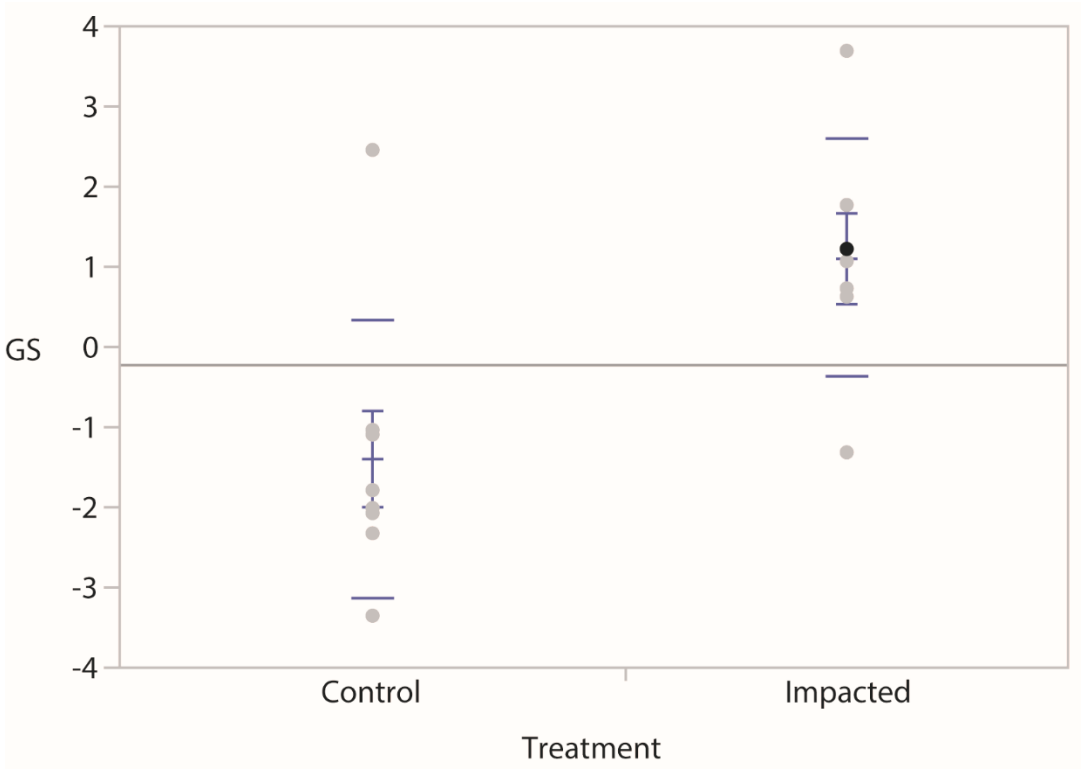


Figure 24. One way analysis of variance (ANOVA) of the GS biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017).

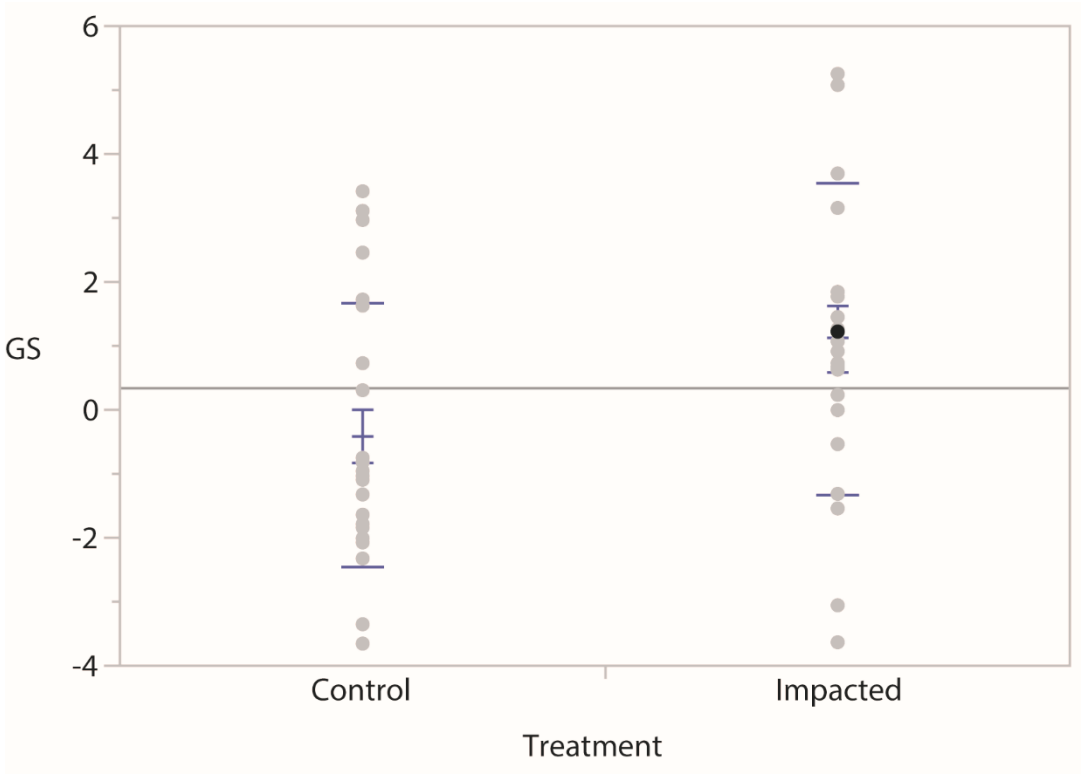


Figure 25. One way analysis of variance (ANOVA) of the GS biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

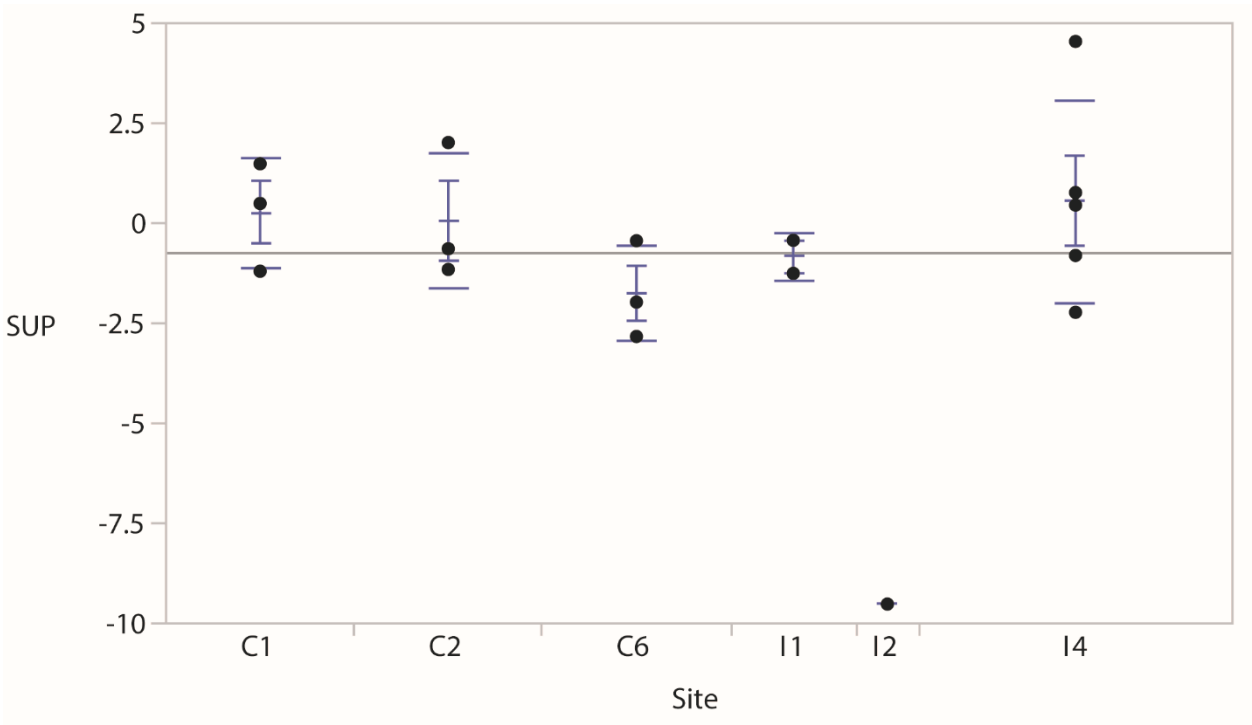


Figure 26. One way analysis of variance (ANOVA) of the RAS biomarker in body tissue of *Crassostrea virginica* between all sites within the spring season (March to April 2017).

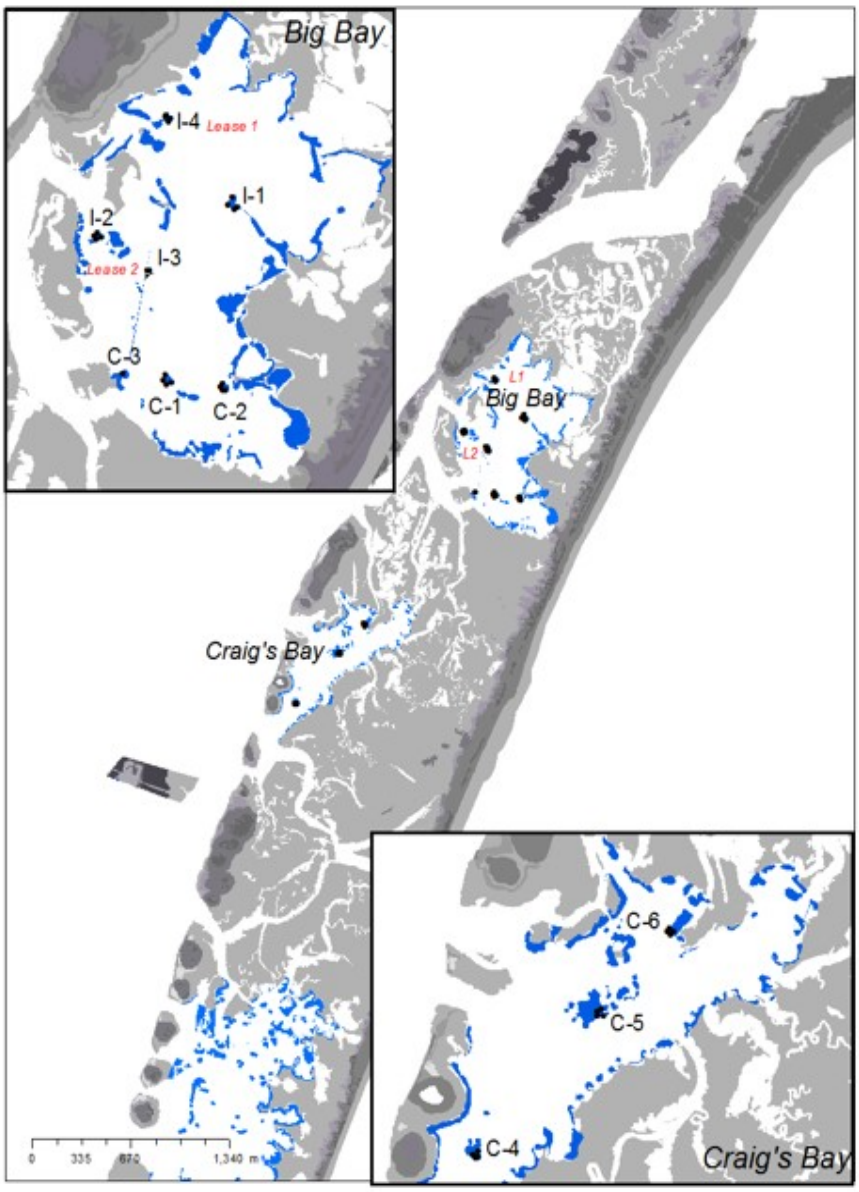
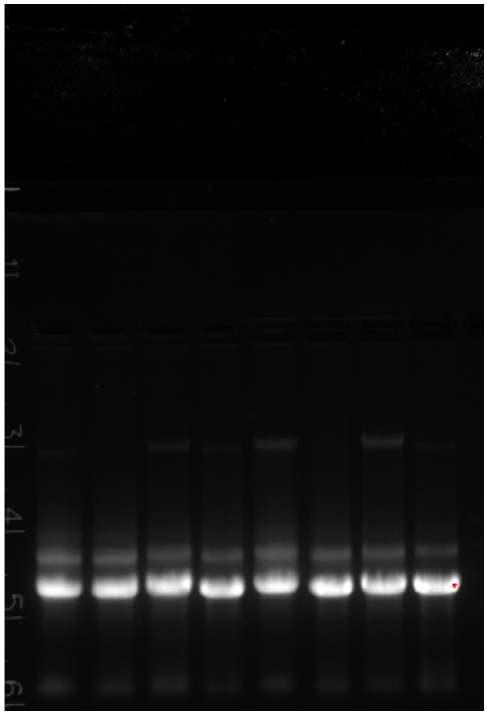


Figure 27. Image of Masonboro Island National Estuarine Research Reserve (NERR) indicating Big Bay as well as Craig's Bay, where each control site (C1-C6) and potentially impacted site (11-I4) were located. The figure also indicates where each of the two oyster leases were located.

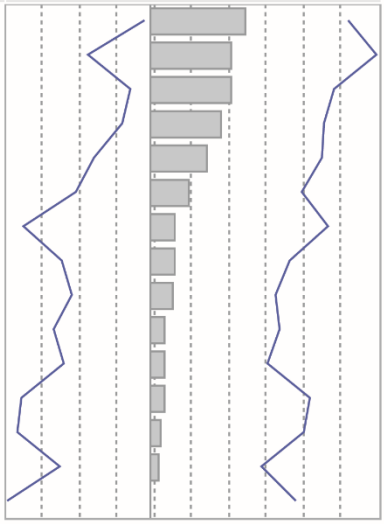
APPENDIX



Appendix A. Image of gel electrophoresis performed 01-03-2018 on RNA samples extracted from oyster body and gill tissue to ensure RNA purity and integrity

Level		Mean
I4	A	2.356722
C1	A	0.885958
C2	A	0.501611
I1	A	0.271638
C6	A	0.268000
I2	A	-0.088361

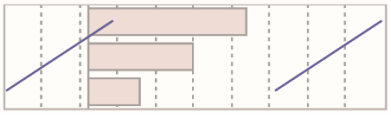
Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
I4	I2	2.445084	0.740204	-0.18424	5.074411	0.0714
I4	C6	2.088722	1.046807	-1.62971	5.807153	0.4118
I4	I1	2.085085	0.740204	-0.54424	4.714412	0.1422
I4	C2	1.855111	0.740204	-0.77422	4.484439	0.2175
I4	C1	1.470764	0.827574	-1.46891	4.410442	0.5215
C1	I2	0.974319	0.827574	-1.96536	3.913997	0.8370
C1	C6	0.617958	1.110307	-3.32603	4.561950	0.9917
C1	I1	0.614320	0.827574	-2.32536	3.553998	0.9709
C2	I2	0.589972	0.740204	-2.03936	3.219300	0.9610
C1	C2	0.384347	0.827574	-2.55533	3.324025	0.9964
I1	I2	0.359999	0.740204	-2.26933	2.989327	0.9955
C6	I2	0.356361	1.046807	-3.36207	4.074792	0.9992
C2	C6	0.233611	1.046807	-3.48482	3.952042	0.9999
C2	I1	0.229973	0.740204	-2.39935	2.859301	0.9995
I1	C6	0.003638	1.046807	-3.71479	3.722069	1.0000



Appendix B. Connecting letters report and ordered differences report for the post hoc Tukey-Kramer for ANOVA on KCrec biomarker in gill tissue of *Crassostrea virginica* between all sites from September to October 2016.

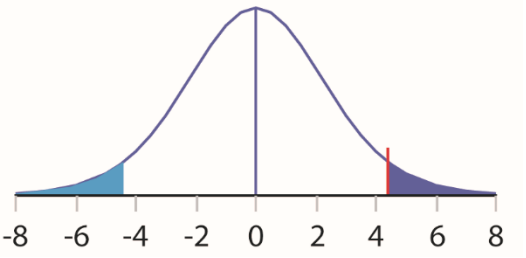
Level			Mean
Fall	A		0.767605
Spring	A	B	-0.108228
Summer		B	-1.846012

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Fall	Summer	2.613616	0.9132508	0.39488	4.832355	0.0176*
Spring	Summer	1.737784	0.9132508	-0.48095	3.956522	0.1504
Fall	Spring	0.875833	0.9132508	-1.34291	3.094571	0.6065



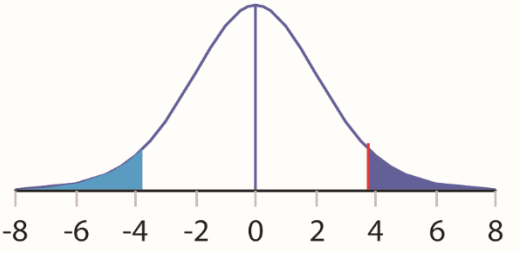
Appendix C. Connecting letters report and ordered differences report for the post hoc Tukey-Kramer for ANOVA on AS6 biomarker in gill tissue of *Crassostrea virginica* between three seasons (fall, spring, and summer).

Difference	4.4020	t Ratio	1.903849
Std Err Dif	2.3121	DF	12.74029
Upper CL Dif	9.4074	Prob > |t|	0.0798
Lower CL Dif	-0.6035	Prob > t	0.0399*
Confidence	0.95	Prob < t	0.9601



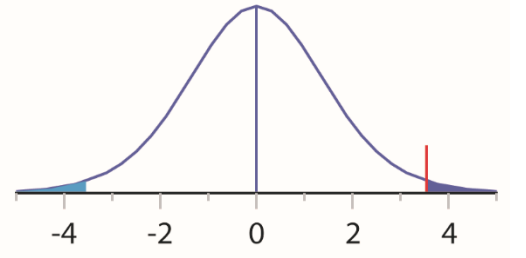
Appendix D. Post hoc Student's t-Test for ANOVA on EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017).

Difference	3.7615	t Ratio	1.758095
Std Err Dif	2.1395	DF	11.69996
Upper CL Dif	8.4363	Prob > |t|	0.1048
Lower CL Dif	-0.9134	Prob > t	0.0524
Confidence	0.95	Prob < t	0.9476



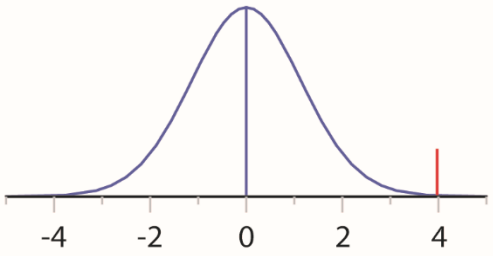
Appendix E. Post hoc Student's t-Test for ANOVA on EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the summer season (June-August 2017).

Difference	3.54619	t Ratio	2.501744
Std Err Dif	1.41749	DF	12.93149
Upper CL Dif	6.61013	Prob > |t|	0.0266*
Lower CL Dif	0.48225	Prob > t	0.0133*
Confidence	0.95	Prob < t	0.9867



Appendix F. Post hoc Student's t-Test for ANOVA on EDL biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the fall season (September to October 2016).

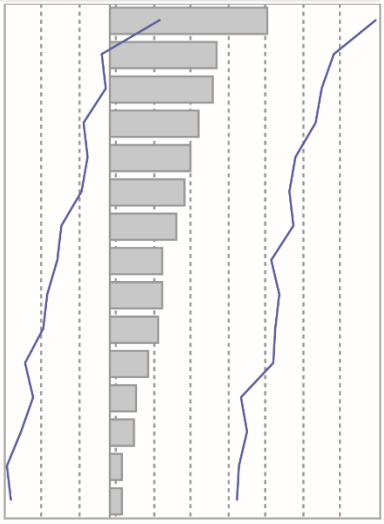
Difference	3.97548	t Ratio	3.439936
Std Err Dif	1.15568	DF	42.98771
Upper CL Dif	6.30615	Prob > |t|	0.0013*
Lower CL Dif	1.64480	Prob > t	0.0007*
Confidence	0.95	Prob < t	0.9993



Appendix G. Post hoc Student's t-Test for ANOVA on EDL biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

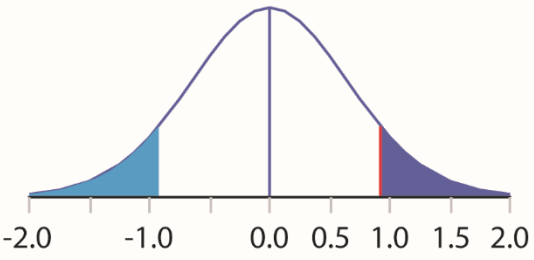
Level			Mean
I1	A		5.378208
I4	A	B	2.561168
I2	A	B	1.840985
C2	A	B	1.123398
C6	A	B	-0.195001
C1		B	-2.803312

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
I1	C1	8.181519	1.842429	2.66162	13.70142	0.0010*
I1	C6	5.573208	1.990050	-0.38897	11.53538	0.0786
I4	C1	5.364479	1.842429	-0.15542	10.88438	0.0610
I2	C1	4.644297	1.990050	-1.31788	10.60647	0.2054
I1	C2	4.254810	1.790519	-1.10957	9.61919	0.1897
C2	C1	3.926710	1.790519	-1.43767	9.29109	0.2641
I1	I2	3.537223	1.990050	-2.42495	9.49940	0.4915
I1	I4	2.817040	1.842429	-2.70286	8.33694	0.6482
I4	C6	2.756168	1.990050	-3.20601	8.71834	0.7355
C6	C1	2.608311	1.990050	-3.35387	8.57049	0.7774
I2	C6	2.035986	2.127453	-4.33785	8.40982	0.9286
I4	C2	1.437769	1.790519	-3.92661	6.80215	0.9653
C2	C6	1.318399	1.942090	-4.50009	7.13689	0.9833
I4	I2	0.720183	1.990050	-5.24199	6.68236	0.9991
I2	C2	0.717587	1.942090	-5.10090	6.53607	0.9990



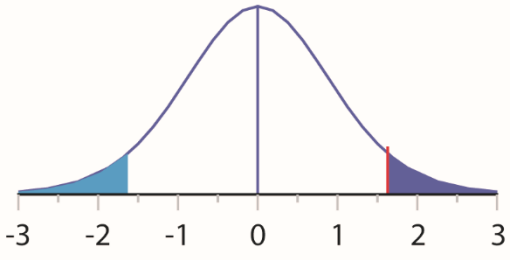
Appendix H. Connecting letters report and ordered differences report from post hoc Tukey-Kramer for ANOVA on EDL biomarker in gill tissue of *Crassostrea virginica* between all sites.

Difference	0.9226	t Ratio	1.402408
Std Err Dif	0.6579	DF	27.79413
Upper CL Dif	2.2707	Prob > |t|	0.1719
Lower CL Dif	-0.4254	Prob > t	0.0859
Confidence	0.95	Prob < t	0.9141



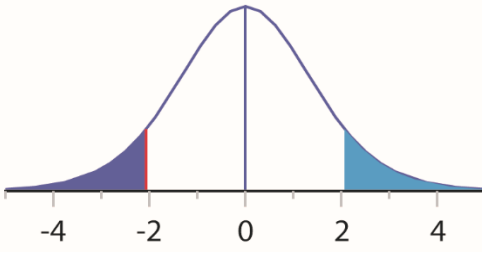
Appendix I. Post hoc Student's t-Test for ANOVA on HSP70 biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

Difference	1.6287	t Ratio	1.777831
Std Err Dif	0.9161	DF	13.92
Upper CL Dif	3.5947	Prob > |t|	0.0973
Lower CL Dif	-0.3372	Prob > t	0.0486*
Confidence	0.95	Prob < t	0.9514



Appendix J. Post hoc Student's t-Test for ANOVA on PRDX6 biomarker in body tissue of *Crassostrea virginica* between control and impacted sites from the fall season (September to October 2016).

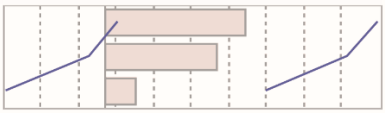
Difference	-2.0667	t Ratio	-1.48286
Std Err Dif	1.3937	DF	10.98357
Upper CL Dif	1.0014	Prob > |t|	0.1662
Lower CL Dif	-5.1348	Prob > t	0.9169
Confidence	0.95	Prob < t	0.0831



Appendix K. Post hoc Student's t-Test for ANOVA on PRDX6 biomarker in body tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017).

Level		Mean
Fall	A	0.919002
Spring	A B	0.437505
Summer	B	-1.356507

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Fall	Summer	2.275508	0.8569592	0.20296	4.348056	0.0284*
Spring	Summer	1.794012	0.8569592	-0.27854	3.866559	0.1019
Fall	Spring	0.481497	0.8569592	-1.59105	2.554044	0.8409

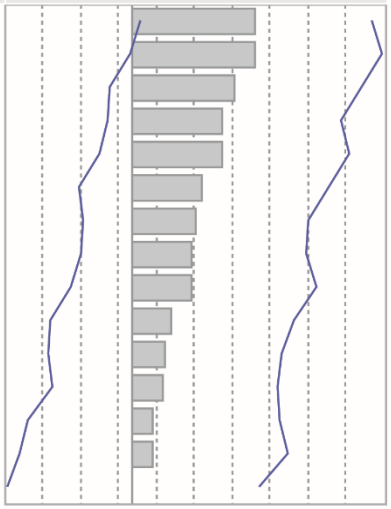


Appendix L. Connecting letters report and ordered differences report from post hoc Tukey-Kramer for ANOVA on PRDX6 biomarker in body tissue of *Crassostrea virginica* between three seasons (fall, spring, and summer).

Level			Mean
I1	A		1.359145
I4	A	B	0.830188
C2	A	B	0.313824
C6	A	B	-0.345307
I2	A	B	-0.689655
C1		B	-0.691416

Ordered Differences Report

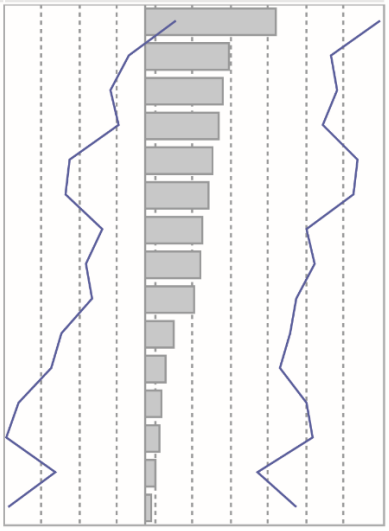
Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
I1	C1	2.050561	0.6440727	0.12092	3.980198	0.0316*
I1	I2	2.048800	0.6956780	-0.03545	4.133046	0.0564
I1	C6	1.704452	0.6956780	-0.37979	3.788698	0.1645
I4	C1	1.521603	0.6440727	-0.40803	3.451240	0.1947
I4	I2	1.519842	0.6956780	-0.56440	3.604088	0.2679
I4	C6	1.175494	0.6956780	-0.90875	3.259740	0.5465
I1	C2	1.045322	0.6259262	-0.82995	2.920592	0.5590
C2	C1	1.005240	0.6259262	-0.87003	2.880510	0.5996
C2	I2	1.003478	0.6789123	-1.03054	3.037494	0.6798
C2	C6	0.659130	0.6789123	-1.37489	2.693146	0.9244
I1	I4	0.528958	0.6440727	-1.40068	2.458595	0.9618
I4	C2	0.516364	0.6259262	-1.35891	2.391634	0.9611
C6	C1	0.346109	0.6956780	-1.73814	2.430355	0.9960
C6	I2	0.344348	0.7437111	-1.88380	2.572501	0.9971
I2	C1	0.001761	0.6956780	-2.08248	2.086007	1.0000



Appendix M. Connecting letters report and ordered differences repor from, post hoc Tukey-Kramer for ANOVA on PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all sites.

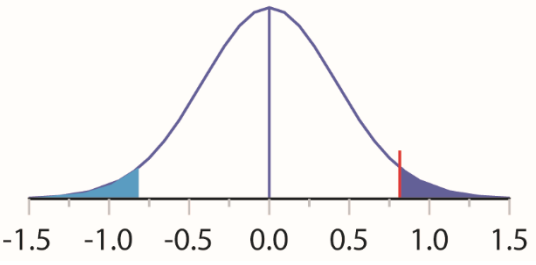
Level			Mean
I4	A		1.945445
I1	A	B	0.529971
C2	A	B	0.242111
C6	A	B	0.053500
C1	A	B	-0.336709
I2		B	-1.944030

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
I4	I2	3.889475	0.850077	0.86986	6.909087	0.0119*
I1	I2	2.474001	0.850077	-0.54561	5.493613	0.1247
I4	C1	2.282153	0.950415	-1.09388	5.658182	0.2498
C2	I2	2.186141	0.850077	-0.83347	5.205753	0.1991
C6	I2	1.997530	1.202190	-2.27285	6.267906	0.5840
I4	C6	1.891945	1.202190	-2.37843	6.162321	0.6323
I4	C2	1.703334	0.850077	-1.31628	4.722946	0.4079
C1	I2	1.607322	0.950415	-1.76871	4.983350	0.5678
I4	I1	1.415474	0.850077	-1.60414	4.435086	0.5820
I1	C1	0.866680	0.950415	-2.50935	4.242708	0.9338
C2	C1	0.578820	0.950415	-2.79721	3.954848	0.9876
I1	C6	0.476471	1.202190	-3.79391	4.746847	0.9983
C6	C1	0.390209	1.275115	-4.13921	4.919626	0.9995
I1	C2	0.287860	0.850077	-2.73175	3.307472	0.9992
C2	C6	0.188611	1.202190	-4.08177	4.458987	1.0000



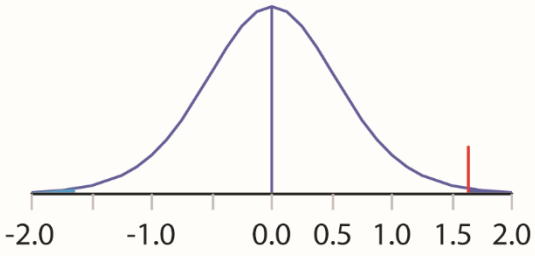
Appendix N. Connecting letters report and ordered differences report from post hoc Tukey-Kramer for ANOVA on PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all sites within the fall season (September to October 2016).

Difference	0.8158	t Ratio	1.92913
Std Err Dif	0.4229	DF	30.71021
Upper CL Dif	1.6786	Prob > |t|	0.0630
Lower CL Dif	-0.0470	Prob > t	0.0315*
Confidence	0.95	Prob < t	0.9685



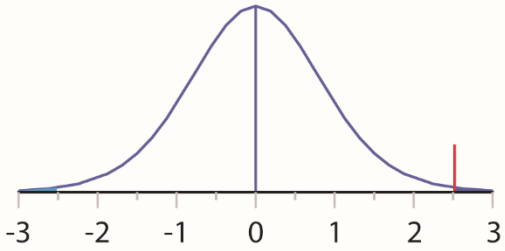
Appendix O. Post hoc Student's t-Test for ANOVA on PRDX6 biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

Difference	1.63850	t Ratio	2.969072
Std Err Dif	0.55186	DF	11.88381
Upper CL Dif	2.84220	Prob > |t|	0.0118*
Lower CL Dif	0.43480	Prob > t	0.0059*
Confidence	0.95	Prob < t	0.9941



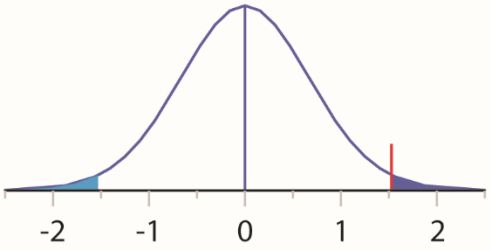
Appendix P. Post hoc Student's t-Test for ANOVA on PRDX6 biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the summer season (June 2017).

Difference	2.51670	t Ratio	3.031605
Std Err Dif	0.83016	DF	12.99997
Upper CL Dif	4.31015	Prob > |t|	0.0096*
Lower CL Dif	0.72326	Prob > t	0.0048*
Confidence	0.95	Prob < t	0.9952



Appendix Q. Post hoc Student's t-Test for ANOVA on GS biomarker in gill tissue of *Crassostrea virginica* between control and impacted sites from the spring season (March to April 2017).

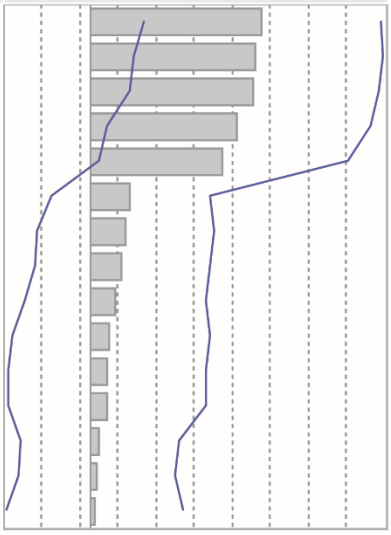
Difference	1.52837	t Ratio	2.268145
Std Err Dif	0.67384	DF	41.20447
Upper CL Dif	2.88902	Prob > |t|	0.0286*
Lower CL Dif	0.16772	Prob > t	0.0143*
Confidence	0.95	Prob < t	0.9857



Appendix R. Post hoc Student's t-Test for ANOVA on GS biomarker in gill tissue of *Crassostrea virginica* between all control and all impacted sites.

Level			Mean
I4	A		0.546172
C1	A		0.259838
C2	A		0.074341
I1	A		-0.840025
C6	A		-1.746230
I2		B	-9.516150

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
I4	I2	10.06232	2.043590	3.09299	17.03166	0.0046*
C1	I2	9.77599	2.154133	2.42966	17.12231	0.0083*
C2	I2	9.59049	2.154133	2.24417	16.93681	0.0095*
I1	I2	8.67613	2.284803	0.88417	16.46808	0.0268*
C6	I2	7.76992	2.154133	0.42360	15.11624	0.0365*
I4	C6	2.29240	1.362393	-2.35382	6.93863	0.5678
C1	C6	2.00607	1.523202	-3.18857	7.20070	0.7708
C2	C6	1.82057	1.523202	-3.37406	7.01521	0.8305
I4	I1	1.38620	1.560818	-3.93672	6.70911	0.9417
C1	I1	1.09986	1.702992	-4.70792	6.90764	0.9845
C2	I1	0.91437	1.702992	-4.89341	6.72214	0.9932
I1	C6	0.90621	1.702992	-4.90157	6.71398	0.9935
I4	C2	0.47183	1.362393	-4.17439	5.11805	0.9991
I4	C1	0.28633	1.362393	-4.35989	4.93256	0.9999
C1	C2	0.18550	1.523202	-5.00914	5.38013	1.0000



Appendix S. Connecting letters report and ordered differences report from post hoc Tukey-Kramer for ANOVA on SUP biomarker in body tissue of *Crassostrea virginica* between all sites within the spring season (March to April 2017).