Louisiana State University
LSU Digital Commons

LSU Master's Theses | Graduate School

10-23-2018

Synergistic Effects of Temperature and Salinity on the Gene Expression and Physiology of Crassostrea virginica

Hollis Jones
Louisiana State University and Agricultural and Mechanical College, hjone52@lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses

Part of the Marine Biology Commons, Molecular Genetics Commons, and the Other Physiology Commons

Recommended Citation
Jones, Hollis, "Synergistic Effects of Temperature and Salinity on the Gene Expression and Physiology of Crassostrea virginica" (2018). *LSU Master's Theses*. 4819.
https://digitalcommons.lsu.edu/gradschool_theses/4819

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

SYNERGISTIC EFFECTS OF TEMPERATURE AND SALINITY ON THE GENE EXPRESSION AND PHYSIOLOGY OF *CRASSOSTREA VIRGINICA*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Biological Sciences

by
Hollis Renee Jones
B.S., Northeastern University, 2015
December 2018

Acknowledgements

I would first like to thank my major advisor, Dr. Morgan Kelly, for her expert guidance throughout the past two and half years and all of my lab mates within the Kelly Lab – Dr. Kevin Johnson, Joanna Griffiths, Kyle Sirovy, and Scott Riley for their support through the project design, execution, and analysis. My thanks also go out to the undergraduate students in the Kelly Lab that helped with the lab work associated with my project – Michelle Gautreaux, Bennett Thomas, Devin Comba, and Yasmeen Kawji.

I would also like to thank my co-advisor Dr. Megan La Peyre for her help designing the project and for her connections with the Louisiana Department of Wildlife and Fisheries who were instrumental to my oyster collection efforts.

I would also like to thank my committee member Dr. Maheshi Dassanayake for her input on my transcriptome assembly. My thanks also go out to Dr. Jerome La Peyre and Dr. Sandra Casas Liste who helped enormously with oyster collection, experimental design, execution, and analysis. This project would not have been possible without them.

Finally, I would like to thank the Louisiana State University Biological Sciences and Renewable Natural Resources Graduate Faculty and Student network that has been instrumental to the completion of this work.

Table of Contents

Acknowledgments……...ii

List of Tables……...……...iv

List of Figures……..……....v

Abstract……...vi

Introduction……..……....1

Materials and Methods…….……....5

Comparative Transcriptomics…….…….……....5

Physiology……...…….……...8

Genes of Interest ……...……..……....9

Results …….…..13

Comparative Transcriptomics……..……..13

Physiology……..……..……16

Genes of Interest……..……..17

Discussion……..21

Synergistic Effects of Temperature and Salinity Stress…….……..……..22

Contrasting Laboratory and Wild Expression…….....……..23

Future Directions……..…….24

Limitations and Considerations……...……..……..……..25

Conclusions……..……..25

Appendix. Oyster Measurements……..…….27

Literature Cited……..29

Vita……...……..35

List of Tables

1. Genes of Interest Sampling Locations Temperature and Salinity Information……..…….....10

2. Enriched Gene Ontology Terms……...……...…..16

3. Genes of Interest……...…….....18

A.1. Oyster Shell Height, Dry Meat Weight, and Individual Physiological Measurements...….27

List of Figures

1. Study Area Map ……...……..…….…....5

2. Experimental Design……...…….……....6

3. Genes of Interest Sampling Locations Daily Mean Salinity and Temperature for 2017……...10

4. Differentially Expressed Gene Counts……...……..……..14

5. Principle Coordinate Analysis of Transcriptomes by Treatment…….....…...……..14

6. Log Fold Change of Genes Responding to Low Salinity at Warm Temperatures……..…..15

7. Box Plot of Respiration and Clearance Rate…….....…….……...17

8. Heat Map of $2^{-\Delta\Delta}$ Values for Genes of Interest……...…….……..19

9. Principle Coordinate Analysis of $2^{-\Delta\Delta}$ Values by Location and Season…….…….…..20

Abstract

Crassostrea virginica, the eastern oyster, forms reefs that provide critical services and benefits to the resiliency of the surrounding ecosystem. Changes in environmental conditions, including salinity and temperature, can dramatically alter the services oysters provide by affecting their population dynamics. Climate warming may further exacerbate the effects of salinity changes as precipitation events increase in frequency, intensity, and duration. Temperature and salinity independently and synergistically influence gene expression and physiology in marine organisms. We used comparative transcriptomics, physiology, and a field assessment experiment to investigate whether Louisianan oyster are changing their phenotypes to cope with increased temperature and salinity stress in Gulf of Mexico. Oysters from Sister Lake, Louisiana were exposed to fully crossed temperature (20°C and 30°C) and salinity (25ppt, 15ppt, and 7ppt) treatments. We found a higher number of genes were differentially expressed (downregulated) in response to low salinity at warmer temperatures – suggesting metabolic suppression. Gene ontology terms for ion transport and microtubule based processes were significantly enriched among upregulated genes in response to low salinity. Ion transport plays a role in osmolyte regulation which is important to maintain cell volume during low salinity. Microtubule based processes play a role in ciliary action which can improve fluid transport, prolonging aerobic metabolism and survival at low salinities. Oyster respiration rate significantly increased between 20°C and 30°C but, despite the higher energetic demands the clearance rate did not comparably increase. To investigate transcriptional differences in wild populations, we collected tissue from three locations across the Louisiana Gulf. We determined the expression levels of seven target genes and found an upregulation of genes that function in osmolyte transport, oxidative stress mediation, apoptosis, and protein synthesis at our low salinity site and sampling time point. Overall, oysters altered their phenotype more in response to low salinity at higher temperatures as evidenced by a higher number of differentially expressed genes during laboratory exposure, increased respiration (higher energetic demands), differentially expression by season and location. Warm temperatures lower the eastern oyster's ability to cope with low salinities; the timing and length of low salinity exposure is important for understanding oyster recruitment, mortality, and growth.

Introduction

Average temperatures are rising in response to a changing global climate; in the northern Gulf of Mexico (GOM) bottom waters experienced an average warming of 0.046˚C/year since 1985 (Turner, Rabalais, & Justić, 2017). Warmer temperatures result in more evaporation and regional changes in precipitation patterns (Biasutti, Sobel, Camargo, & Creyts, 2012). The Continental United States has experienced recent droughts in the West, while Southeastern states have been experiencing record wet years (Blunden, Arndt, Blunden, & Arndt, 2016). This rise of precipitation in the Southeastern states combined with increased snow and ice melt has led to greater freshwater outflow from two large rivers that flow into the GOM through the state of Louisiana. The Mississippi and Atchafalaya Rivers account for over 80% of the total freshwater input to the Gulf of Mexico (NOAA, 2016), resulting in Louisiana's coast having the lowest salinities in the GOM (NOAA, 2016). Warming water temperatures and increased freshwater input are of special concern for aquatic invertebrates in the northern GOM because simultaneous stressors may have greater effects than either stressor experienced individually.

Environmental conditions can change rapidly and simultaneously along multiple axes, resulting in shifts in species distribution limits and lethal tolerances as well as constraining both plastic and evolutionary responses to global climate change (DeBiasse & Kelly, 2016; Kelly, Pankey, Debiasse, & Plachetzki, 2016; Somero, 2010). Most marine invertebrates are osmoconformers with internal osmolarities close to that of the surrounding seawater, which allows them to tolerate a wide salinity gradient (La Peyre, Eberline, Soniat, & La Peyre, 2013) while simultaneously making them sensitive to environmental changes in salinity (Zhao, Yu, Kong, & Li, 2012). For instance, exposure to acute or long term low salinity causes mass mortality in osmoconforming marine invertebrates (Gardner & Thompson, 2001; Laing, 2002; Y. Li, Qin, Abbott, Li, & Benkendorff, 2007; X. L. Meng, Dong, Dong, Yu, & Zhou, 2011; Rybovich, Peyre, Hall, & Peyre, 2016). La Peyre (2013) observed that the marine invertebrate *Crassostrea virginica* was able to survive low salinity exposure (below 3ppt) when temperatures were under 25˚C but experienced negative impacts on recruitment, survival, and growth in extended low salinities during the hot summer months (La Peyre et al., 2013). Marine invertebrates are also affected by temperature, a factor that strongly influences distribution, biology, and physiology (Guzman & Conaco, 2016; Lancaster et al., 2016; Runcie et al., 2012; Schoville, Barreto, Moy, Wolff, & Burton, 2012). The combined effects of temperature and salinity on osmoconforming marine invertebrates result in constraints that can partially stem from elevated energetic demands during stress exposure which manifest as increased physiological activity, cellular maintenance, and damage repair (A. Li, Li, Song, Wang, & Zhang, 2017; Sokolova, 2013; Somero, 2002). These cellular functions can compete with other functions such as reproduction and growth as well as lowering the organism's ability to buffer against fluctuations in food availability, resulting in energetic tradeoffs (Y. Li et al., 2007) controlled by changes in gene expression (Eierman & Hare, 2014; Lauritano, Procaccini, & Ianora, 2012; Brent L. Lockwood & Somero, 2011).

Tradeoffs occur when two traits or functions cannot be simultaneously optimized, and may constrain an organism's plastic response to stress (Kelly, DeBiasse, Villela, Roberts, & Cecola, 2016; Sokolova, Frederich, Bagwe, Lannig, & Sukhotin, 2012). Kelly et al. (2016a) found that when salinity tolerant selected lines of *Tigriopus californicus* were exposed to heat stress at ambient salinities their heat tolerance was not diminished; however, when they were simultaneously exposed to heat and hypo-osmotic stress their heat tolerance was decreased.

Further, they conclude that this observed physiological tradeoff is driven by competing energetic demands and not a shared stress response pathway as there was little overlap in the transcriptomic responses to the stressors (Kelly, Pankey, et al., 2016). Li et al. (2017) observed two subspecies of *Crassostrea gigas* under high temperature stress and reported energy limiting stress tolerance. They observed differences in basal and induced responses to stress and a switch from aerobic and anaerobic metabolism resulting in physiological and energetic consequences.

The combination of temperature and salinity stress can have non-additive, synergistic effects on marine invertebrate physiology and gene expression (A. Li et al., 2017; R. Przeslawski, Davis, & Benkendorff, 2005). For example, heat stress can induce a shift from aerobic to anaerobic metabolic processes that can lead to a buildup of toxic end products (Sokolova et al., 2012) or a downregulation of genes involved in energy metabolism to alleviate potential oxidative damage from elevated amounts of metabolic byproducts (A. Li et al., 2017). In addition to heat stress, low environmental salinities result in metabolically costly active transport of inorganic ions such as Na^+, Cl^-, and K^+ (Eierman & Hare, 2014; Sokolova et al., 2012). Studies such as these demonstrate that multiple stressors can lead to competing demands and energetic tradeoffs between low salinity induced ion regulation and heat induced metabolic demands. The synergistic effects of combined temperature and salinity stress on oyster physiology can cause shifts in lethal tolerances and lead to competing energetic demands and tradeoffs.

Crassostrea virginica, the eastern oyster, is an ecologically, economically, and culturally important marine invertebrate inhabiting estuaries along the North American Atlantic and Gulf coasts (NOAA, 2007). Changes in the eastern oyster's environment are impacting their survival, reproduction, and growth (Dickinson et al., 2012; Lowe, Sehlinger, Soniat, & La Peyre, 2017). Over 80% of oyster reefs have been lost globally (Grabowski et al., 2012) and we have seen recent dramatic declines in Louisianan *C. virginica* populations. The Louisiana Department of Wildlife and Fisheries (LDWF) 2014 oyster stock assessment of 1,881,114 barrels was 44% below the 1982-2013 long-term average of 3.3 million barrels. This is due to overfishing, disease, and loss of suitable habitat, which is caused in part by lower environmental salinities due to increased precipitation and river outflow. Additionally, in Louisiana, river diversions also play a role in decreased salinity. The 2010 Deepwater Horizon oil spill resulted in several freshwater diversions during the hot summer months in order to prohibit surface oil from reaching the marshes but, severely reduced spawning stock, larval production, and spat settlement for that year which subsequently reduced the number of market sized oysters in the following years (LDWF, 2016).

The Louisiana eastern oyster fishery is responsible for 45% of the national total (LDWF, 2016), but aside from their value as a fishery, the ecosystem services that they provide such as shoreline stabilization, nursery habitat, and water filtration have been valued at between $5,500 and $99,000/ha/year (Grabowski et al., 2012). The northern GOM states lead the United States in eastern oyster production; therefore, understanding how shifts in temperature and salinity will impact populations is important for regionally specific conservation efforts. Understanding small-scale populations differences in *C. virginca*'s stress response may also lead to more effective oyster reef restoration projects (Leonhardt, Casas, Supan, & La Peyre, 2017).

Defining optimal conditions is intrinsically complex and will change depending on scale, local adaptation or acclimatization, and body size (Lowe et al., 2017). The Louisiana Department of Wildlife and Fisheries reports optimal salinities for growth between 18 – 25 ppt but, Lowe et al (2017) used 40 years of monitoring data to estimate that the optimal combination of

temperature and salinity for growth for all size classes of Louisianan oysters (spat, seed, and sack) and found it to be between 20 – 26.3 ˚C and 10.7 – 16.1ppt. Warmer waters facilitate growth so these 'optimal' temperatures may shift to be cooler when growth is not the metric of interest. Additional factors to consider when designing optimal condition windows will be that in sustained high salinities oyster mortality increased from oyster drill (*Stramonita haemastoma*) predation (Rybovich et al., 2016) and *Perkinsus marinus* infection (La Peyre, Nickens, Volety, Tolley, & La Peyre, 2003).

Rybovich et al (2016) observed significantly different mortality of spat, seed, and market-sized oysters at 25˚C and 32˚C across the salinities 1, 5, and 15 ppt. They observed that oysters across all three size classes at 5ppt had greater than 50% mortality at 32˚C an less than 40% mortality at 25˚C (Rybovich et al., 2016). Lowe et al (2017) found that prolonged exposure to temperatures above 30˚C impacted seed and sack growth and mortality (Lowe et al., 2017). The LDWF also reports that salinities below 5ppt almost completely inhibits growth (LDWF, 2016). Oysters' ability to buffer against environmental stressors changes with season because of reproductive stage; gametogenesis is energetically taxing and they spawn twice a year (late spring and early fall) with 25˚C as the critical temperature for stimulating gamete production (LDWF, 2016).

Because oysters are able to tolerate prolonged and acute (below 5ppt) low salinity at lower temperatures (such as 20˚C) the timing of intense precipitation events is becoming more important. Oysters are unable to properly osmoregulate during high temperatures so they can close their valves to reduce the high energetic demands of osmoregulation during high temperatures but closing their valves for long periods of time can result in mortality from lack of oxygen, buildup of toxic end products, or starvation (La Peyre et al., 2013). The risk of mortality at low salinities depends heavily on how long the low salinity event lasts and whether it happens during a period of high temperatures.

The effects of temperature and salinity on *Crassostrea virginica*'s growth and mortality (La Peyre et al., 2013; Lowe et al., 2017; Rybovich et al., 2016), physiology (Casas, Lavaud, et al., 2018; Heilmayer, Digialleonardo, Qian, & Roesijadi, 2008), and gene expression (Chapman et al., 2011) have individually been documented, but there is a growing need to make links between these phenotypic changes and physiological response in order to fully understand the synergistic effects of multiple stressors. In order to explore the eastern oyster's response to combined temperature and salinity stress we used a three-pronged approach. Multiple stressors interact in non linear ways and gradual changes may produce sudden and unpredictable responses that may manifest themselves in unexpected ways (Harley et al., 2006).

Our work uses a combination of comparative transcriptomics, physiological measurements, and field validation through qPCR to understand the synergistic effects of combined temperature and salinity stress. Oysters experience the highest temperatures and the lowest salinities during the same timeframe, June to August (Figure 3). To quantify the molecular response to temperature an salinity stress we sequenced the transcriptomes of oysters from Sister Lake, Louisiana that were exposed to fully crossed temperature (20°C and 30°C) and salinity (25ppt, 15ppt, and 7ppt) treatments. Gene expression analysis can also help us understand differences between individuals and populations that may be happening very quickly and are not reflected other observable metrics of their physiology (Evans & Hofmann, 2012). Sister Lake, Louisiana has 2006 – 2016 average temperatures of 22.8˚C ± 5.4 ˚C and salinity 12.2 ± 5.4 ppt (USGS continuous data recorder #7381349). Both 20˚C and 30˚C are regularly experienced by oysters at this location but 30˚C is close to the maximum temperature and the

point at which they start to experience declines in physiological functioning. We did not want the oysters to close their valves during the experiment so we chose 7ppt salinity as our stressful salinity treatment. We chose 15ppt salinity because it falls within optimal condition windows and should not negatively affect physiological functioning and while 25ppt is never experienced by these oysters we chose it as well to mimic high salinity estuaries found in Louisiana and, it falls within the LDWF optimal growth window.

Second, we took physiological measurements of respiration and clearance rates for oysters at both temperatures (20˚C and 30˚C) but only at the lowest salinity (7ppt) so that we could analyze their energetic response at treatments that mimic summer conditions. Third, we conducted a field validation experiment to identify differential expression of genes putatively signaling for combined temperature and salinity stress for a fine scale comparison of transcriptional differences across three sites on the Louisiana Gulf coast: Calcasieu Lake, Vermilion Bay, and Sister Lake. Calcasieu Lake has 2006 – 2016 average temperatures of 22.2 ± 6.9 ˚C and average salinities of 19.8 ± 5.6 ppt and will be considered our intermediate high salinity site (USGS continuous data recorder #8017118). Vermilion Bay has 2006 – 2016 average temperatures of 21.7 ± 7.0 ˚C and average salinities of 4.2 ± 3.1 ppt and will be considered our low salinity site (USGS continuous data recorder #7387040). As mentioned earlier, Sister Lake, Louisiana has 2006 – 2016 average temperatures of 22.8˚C ± 5.4 ˚C and salinity 12.2 ± 5.4 pp and will be considered our intermediate low salinity site.

We were especially interested in how physiological data lines up with transcriptomic data and whether observed differences in the molecular response in the lab translate to wild oyster expression.

Materials and Methods

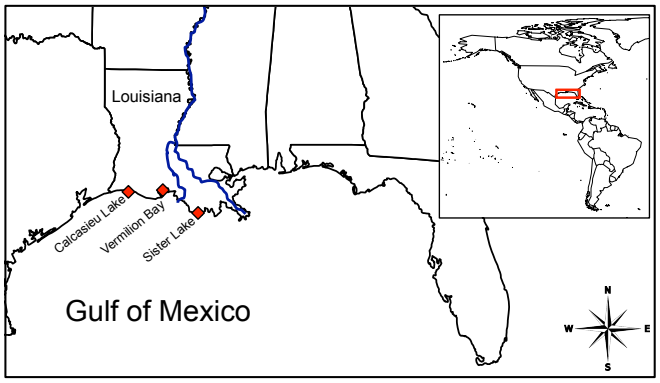

Figure 1. Study Area Map.
Map of the study area indicating where the laboratory exposed oysters were collected (Sister Lake), and the three sites where wild oysters were collected in the summer of 2017: Calcasieu Lake, Vermilion Bay, and Sister Lake. The Mississippi and Atchafalaya Rivers are shown in blue with the Atchafalaya River as the western bifurcation of the Mississippi River.

Comparative Transcriptomics

Oyster Collection and Laboratory Exposure: 2015 & 2017

We collected oysters for transcriptomic sequencing at two time points. First, in August 2015; ~350 oysters were dredged from Sister Lake, Louisiana (29º14'57" N, 90º55'16" W) (See Figure 1) and maintained at Louisiana Sea Grant's Oyster Research Lab in Grand Isle, Louisiana. All oysters were estimated to be around 1.5 years old and 70.3 ± 7.2 mm in shell height (SH), as determined as part of a larger study (Casas, Filgueira, et al., 2018). After 3 months of acclimation at the hatchery, in October 2015, these oysters were transported to the Louisiana State University Agricultural Center Animal and Food Sciences laboratory building (AFL) in Baton Rouge, LA for exposure. The exposure took place at the end of Fall 2015 so that oysters would be in a resting gonadal stage.

The second collection was in April 2017, when ~100 oysters were dredged from Sister Lake, Louisiana and directly transported to the AFL for acclimation and exposure. Shells were 97.7 ± 13.2 mm SH.

For both collection dates oysters were pressure washed, scrubbed, and all biofoulers (barnacles and mussels) were removed with a knife. When exposure began, the salinities and temperatures were adjusted at a rate of three units per day, every other day until they arrived at their experimental values. The 2015 oysters were exposed to fully crossed temperature treatments of 20°C (cool) and 30°C (warm) and salinity treatments of 15ppt (medium) and 25ppt (high). The 2017 oysters were exposed to fully crossed temperature treatments of 20°C (cool) and 30°C (warm) and a single salinity treatment of 7ppt (low) (See Figure 2). Oysters were kept in 700-L tanks with recirculating artificial seawater.

Oysters were kept in experimental conditions for four weeks and fed 0.5ml of Shellfish Diet 1800, a commercial mix of six marine microalgae, (Reed Mariculture, Campbell, CA, USA) per oyster daily (calculated based on manufacturer suggestions). Temperature and salinity were

checked daily using a YSI (YSI model 85, YSI Inc., Yellow Springs, OH, USA) and thermometers. Salinity was maintained using aerated and carbon filtered freshwater (0.5µm carbon filter) and artificial seawater made using Instant Ocean (Spectrum Brands, Madison, WI, USA). Temperature in the isothermal rooms at 20˚C and 30˚C was maintained using air conditioning units and space heaters; the entire room was cooled or heated to produce water temperatures of 20˚C or 30˚C so that there were no temperature gradients in the 700-L tanks.

After four weeks at these treatments four haphazardly selected oysters from each treatment (16 oysters from the 2015 exposure and 8 oysters form the 2017 exposure: 24 total oysters) were sacrificed and 4mm x 4mm gill tissue samples were excised and stored in RNA*later* (Thermo Fisher Scientific, Waltham, MA, USA) for RNA-seq library prep.

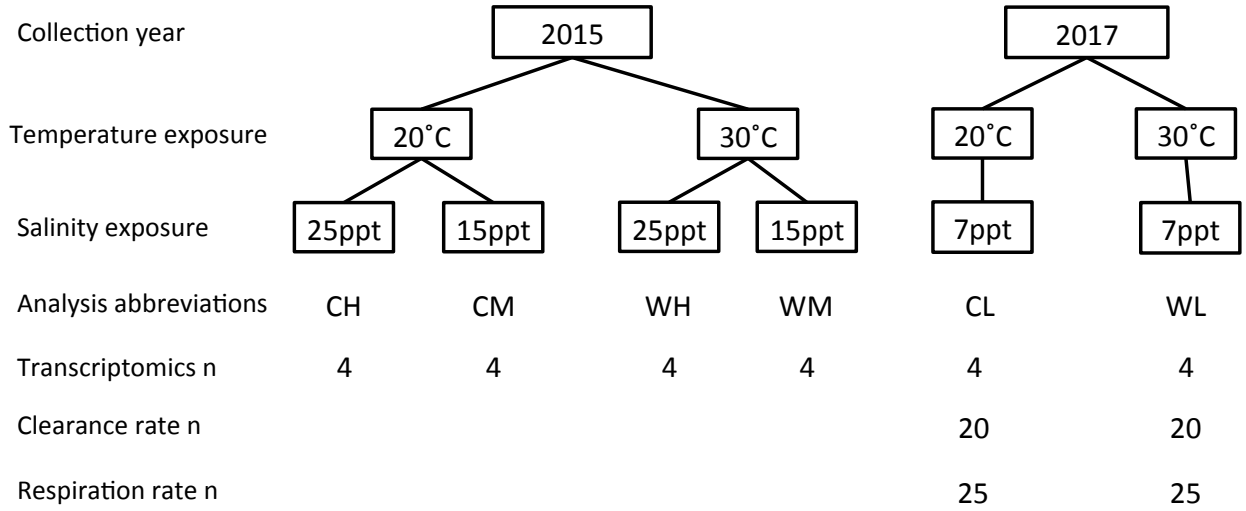

Figure 2. Experimental Design.
Experimental design including both *Crassostrea virginica* sampling years (2015, 2017), the laboratory temperature (C: 20˚C, W: 30˚C) and salinity (H: 25ppt, M: 15ppt, L: 7ppt) exposures, treatment abbreviations used to describe further analyses, and sample sizes for transcriptomics, clearance rate, and respiration rate.

RNA-seq Library Preparation, Sequencing, and Quality Filtering

Twenty-four oysters were used in the RNA-seq analysis, four from each temperature and salinity combination (16 from 2015 and eight from 2017). Total RNA was extracted with E.N.Z.A. Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA). A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the concentration of RNA and 500ng of total RNA starting material was used for Illumina sequencing libraries for consistent concentrations of starting RNA across all samples. Messenger RNA (mRNA) was isolated from total RNA using NEBNext Poly(A) mRNA Magnetic Isolation kit (New England Biolabs, Inc., Ipswitch, MA, USA) and sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Inc.). Fragment size ranges and library concentrations were determined using the Agilent 2100

Bioanalyzer high-sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA). Final library concentrations were quantified using the NEBNext Library Quant Kit Quick Protocol (New England Biolabs, Inc.) and the ViiA-7 Real-Time PCR System (Thermo Fisher Scientific). The KAPA Library Quantification Kit Data Analysis Template (Kapa Biosystems, Inc., Wilmington, MA, USA) was used to calculate Illumina sequencing pooling volumes. Multiplexed libraries were sequenced across three lanes of an Illumina HiSeq3000 sequencer (twelve 100bp paired-end libraries per lane for the 2015 samples and eight 100bp singe-end libraries per lane for the 2017 samples) (Illumina, Inc., San Diego, CA, USA) at the Iowa State University's DNA Sequencing Facility (Ames, IA, USA).

Reads were quality checked using FastQC (v0.11.5) (Andrews, 2010) and trimmed as needed for adapters using Trimmomatic (v0.38) (Bolger, Lohse, & Usadel, 2014). Reads shorter than 36 bp were discarded.

Transcriptome Assembly, Read Mapping, and Differential Expression

Quality controlled single-end reads were mapped to the published transcriptome for *C. virginica* (Gómez-Chiarri, Warren, Guo, & Proestou, 2015) using RSEM (v.1.3.0) (B. Li & Dewey, 2011). Using all high quality reads from the 2015 oysters, a genome-guided de novo transcriptome was also assembled using the published *C. virginica* genome available on NCBI (Gómez-Chiarri et al., 2015) and de novo assembler Trinity (v2.3.2) (Haas et al., 2014). This transcriptome was created to look for Louisiana specific differences in the sequences, as the oyster used to make the published genome and transcriptome is from the coastal Eastern United States. This transcriptome assembly was not used in any of the mapping for differential gene expression analyses but only for qPCR primer design.

Differentially expressed genes were identified using the edgeR (v3.20.7) package (Robinson, McCarthy, & Smyth, 2009) in R (v3.4.2) (R Development Core Team, 2013) using a False Discovery Rate (FDR) cut-off of 0.05. Individual oyster libraries were identified by their treatment temperature (30˚C as W and 20˚C as C) and treatment salinity (25ppt as H, 15ppt as M, 7ppt as L). Genes were removed that did not have at least one count per million in at least three samples. Read counts were normalized by their library size and each transcript was fit to a general linearized model (GLM) for subsequent pairwise comparisons between treatment groups. Pairwise comparisons were done using the glmLRT function that considers a generalized linear model of the negative binomial distribution family to test for significant differential expression. Using the abbreviations defined in Figure 2, we made the following nine comparisons: WH vs. WL, WH vs. WM, WM vs. WL, CH vs. CL, CH vs. CM, CM vs. CL, CH vs. WH, CM vs. WM, and CL vs. WL. We assessed overall patterns of expression by comparing the number of genes upregulated or downregulated (Figure 4). We also plotted the log fold changes of all significantly differentially expressed genes between pairwise comparisons and compared the number of genes that fell below or above the one to one line using a Fisher's exact test (Figure 6).

We performed a principle coordinate analysis (PCoA) by calculating Euclidean distances in the R package vegan to visualize clustering of gene expression between the two temperature treatments (warm and cool) and the three salinity treatments (high, medium, and low) (Figure 5). Significance was calculated using a permutational MANOVA using the adonis function in the R program vegan (2.5.2) (Anderson, 2001).

Annotation and Functional Enrichment Analysis

The differentially expressed transcripts were annotated using sequence annotations available with the latest release of the *C. virginica* genome (GCF_002022765.2, version 3.0). These genome annotations were combined with gene ontology (GO) terms generated using InterProscan 5 (v 5.27-66.0) as described by Johnson et al (2018) (*in review*).

After annotation, we were able to compare individual responses to look for overlap in genes that were upregulated or downregulated between treatments. We performed a Mann-Whitney U (MWU) test in R (v3.4.2) using the package ape to look for up or downregulated functionally enriched biological process (BP) GO terms in our pairwise comparisons (R Development Core Team, 2013; Wright, Aglyamova, Meyer, & Matz, 2015). Further information, scripts, and examples files are available at https://github.com/z0on/GO_MWU.

Physiology

Oyster Collection and Laboratory Exposure

For the oysters collected in Spring 2017 and exposed to the treatment temperatures 20˚C and 30˚C and the low salinity treatment 7ppt that were not sacrificed for RNA-seq analysis, we measured clearance rate (L h^{-1}) (n=40) and respiration rate (mg O_2 h^{-1}) (n=50) in summer 2017. Gonadal development was not measured. For the oysters used for respiration rate measurements we also measured dry meat weight (g) to standardize the rates. Dry body weight was measured after drying at 70˚C for 48 hours. For the oysters used for clearance rate measurements, shell heights (mm) were used to standardize the rates. Individual clearance rates, respiration rates, shell heights, and dry meat weights are reported in Appendix 1.

Oysters used for clearance rate and respiration rate measurements were starved for one-week prior to measurement to ensure that we measured their standard or basal metabolic rates (Shumway & Koehn, 1982). Because RNA-seq tissue sampling is lethal and this starvation period can affect gene expression, we were not able to use the same oysters for both RNA-seq and the physiological measurements of respiration rate and clearance rate.

Respiration Rate

Respiration rate (mg O_2 h^{-1}), or oxygen consumption, was measured for 25 individual oysters from each of the two temperature treatments and 7ppt salinity (a total of n=50). Individuals were held in 0.915L acrylic respirometers, closed except for an oxygen probe opening, in the isothermal rooms using self-stirring YSI ProOBOD probes (YSI Incorporated) with calibrated optical dissolved oxygen and temperature sensors. The changes in oxygen solubility due to salinity were accounted for by manually entering the measured salinity. The rate of decline of oxygen concentration (mg/L) was recorded every 30 seconds using a YSI Multilab 4010-3 (YSI Incorporated). The recording period was ended when the oxygen percentage fell below 70% saturation. Respiration rate was calculated using the equation: Rate of O_2 uptake (mg O_2 h^{-1}) = [(b-b') x Vol(L) x 60 min h^{-1}] where b is the slope of the linear regression of oxygen concentration (mg L^{-1}) by time, b' is the slope of the control, and Vol is the volume of water in the respirometer. Respiration rate was standardized using dry body weight (g).

We tested for significance using a one-way ANOVA of either respiration or clearance rate against temperature in R (v3.4.2) (R Development Core Team, 2013) (Figure 7).

Clearance Rate

Clearance rate (L h^{-1}), the volume of water cleared of suspended particles per unit of time, was measured for 20 individual oysters from each of the two temperature treatments and 7ppt salinity (a total of n=40) following the methods of Casas et al (2017). Oysters were 98.7 ± 12.7 mm SH. Temperature was controlled by heating or cooling the entire room that held the 700L tanks with the oysters in them so they were considered 'isothermal rooms'. Clearance rate was measured within the isothermal rooms in 0.5 µm filtered seawater from the oyster holding tanks so that no temperature or salinity changes were experienced by the oysters. Individuals were placed in 2L glass beakers with a single air stone on low with a beginning algae concentration of 30,000 cells/mL. 25ml of seawater was sampled every ten minutes for forty minutes and algae concentrations were calculated at each time period using a particle size counter (Multisizer 3 Beckman Coulter Counter, 100 µm pore orifice, particle threshold >4.5 µm). Clearance rate was calculated using the equation: CR L h^{-1} = [(b-b') x Vol (L) x 60 min h^{-1}] where b is the slope of the linear regression between ln cells mL^{-1} and time, b' is the slope of the control, and Vol is the volume of the seawater in the beaker (2L). Clearance rate was standardized using a 100mm shell height. Only clearance rates with a linear regression of r^2 > 0.90 were included in the subsequent analyses.

Genes of Interest

Oyster Collection

In the summer of 2017 we excised and stored *C. viginica* gill tissue in RNA*later* from adult oysters in the wild. Individuals were manually dredged from Sister Lake on two different time points (A and B) (A: n=15, B: n=15), Vermilion Bay (n=15), and Calcasieu Lake (n=16) Louisiana (See Figure 1). We had two collection dates for the Sister Lake population (A: April 25, 2017 and B: July 6, 2017) and single collection dates for Vermilion Bay (June 27, 2017) and Calcasieu Lake (June 19, 2017) (See Table 1). Tropical Storm Cindy made landfall in southwest Louisiana on June 22, 2017 resulting in heavy rainfall across the northern GOM coast and lowering the salinity for the sampling events in the days following. The tissue was excised and stored within minutes of dredging so that there were minimal changes in gene expression. The tissue from these oysters was used in a quantitative PCR analyses to test for variation in gene expression among sites and sampling times that differed in salinity and temperature.

Table 1. Genes of Interest Sampling Locations Temperature and Salinity Information. A) Mean annual temperature and salinity ± SD for Calcasieu Lake, Vermilion Bay, and Sister Lake, calculated as daily averages over a ten year period (1/1/06 to 1/1/16) using data from USGS sites located as close to the oyster beds as possible. B) Mean temperature and salinity ± SD for the month preceding the sampling date for the four wild oyster collections at Calcasieu Lake, Vermilion Bay, and the two Sister Lake collections. Calculated as daily averages using data from USGS sites located as close to the oyster beds as possible. Calcasieu Lake: USGS ID 08017118, Latitude 29˚48'56", Longitude 93˚20'56". Vermilion Bay: USGS ID 07387040, Latitude 29˚42'47", Longitude 91˚52'49". Sister Lake: USGS ID 07381349, Latitude 29˚14'57", Longitude 90˚55'16".

A)

	Calcasieu Lake	Vermilion Bay	Sister Lake
Temperature (˚C)	22.2 ± 6.9	21.7 ± 7.0	22.8 ± 5.4
Salinity (ppt)	19.8 ± 5.6	4.2 ± 3.1	12.2 ± 5.4

B)

	Calcasieu Lake	Vermilion Bay	Sister Lake (A)	Sister Lake (B)
Sampling Date	6/19/17	6/27/17	4/25/17	7/6/17
Temperature (˚C)	27.4 ± 1.1	27.6 ± 1.2	24.1 ± 1.2	29.0 ± 1.3
Salinity (ppt)	11.4 ± 4.1	0.8 ± 0.3	18.3 ± 2.0	5.0 ± 2.6

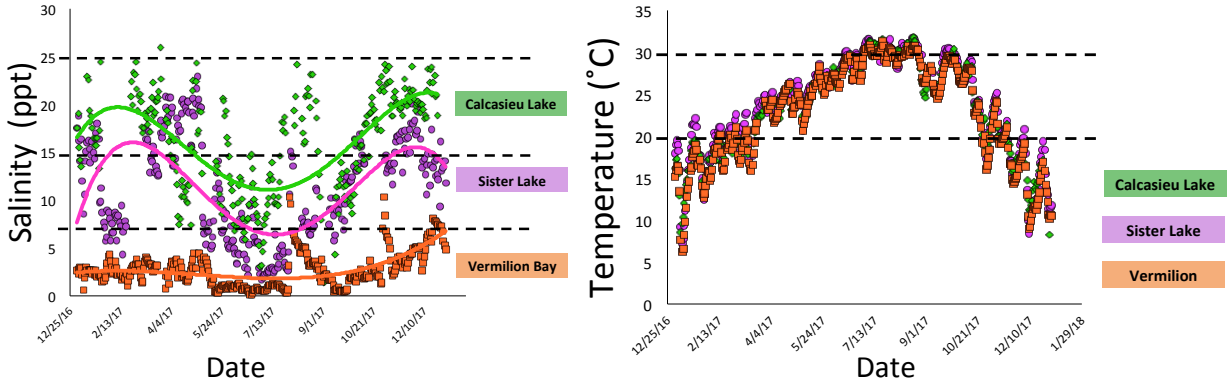

Figure 3. Genes of Interest Sampling Locations Daily Mean Salinity and Temperature for 2017. Mean daily salinity (ppt) and temperature (˚C) for Calcasieu Lake, Vermilion Bay, and Sister Lake for the year 2017. Treatment salinities (7, 15, and 25 ppt) and temperatures (20 and 30 ˚C), used for the comparative transcriptomics and physiological experiments, are marked by perforated black lines.

Target Genes: Selection and Primer Design

Expression of seven target genes (carnosine synthase, elongation factor 1-alpha, heatshock protein 70, anti-apoptotic protein, cadherin, palmitoyltransferase, and sodium hydrogen exchanger) and one housekeeping gene (myosin light chain kinase) was measured. We

selected our genes of interest based on several lines of inquiry. We chose heatshock protein 70 (HSP 70), a molecular chaperone, and anti-apoptotic protein, which is key for survival in highly variable environments, because of their well-studied role in the stress response (Zhao, Yu, Kong, & Li, 2012). We chose a sodium hydrogen exchanger because of its role in transporting the osmolyte Na^+ (Huth & Place, 2016). Carnosine synthase (Boldyrev, Aldini, & Derave, 2013), cadherin (Joshi et al., 2005), and palmitoyltransferase (Zaugg et al., 2011) were chosen by looking at annotated lists of highly differentially expressed genes between all of the pairwise comparisons; all three were highly differentially expressed in at least two different comparisons and all play a role in mediating oxidative stress (Table 3). We originally chose elongation factor 1-alpha (EF1A), a protein synthesis gene, as our housekeeping gene based on results in (Du et al., 2013) but found that it had a lot of variation between locations so repurposed it as a target gene. Myosin light chain kinase has been previously shown as a steady housekeeping gene in *Tigriopus californicus* studies (Schoville et al., 2012), as well as in our transcriptomic data, so we then made it our housekeeping gene. Across the nine edgeR comparisons myosin light chain kinase showed an average log fold change of 0.09 ± 0.41, p value of 0.48 ± 0.23, and false discovery rate of 0.98 ± 0.04.

qPCR primers were designed in Geneious® 11.1.2 using NCBI published transcriptome sequences as well as the Trinity *de novo* assembled transcriptome to capture Louisiana specific sequences. Oligonucleotides were ordered through Eurofins Genomics LLC. Primers were diluted in 1X TE Buffer to 100µM and then diluted again in nuclease free water to 1:10. Each primer set was tested on three oyster individuals, each from a different Louisianan population using *Taq* PCR Kit (New England Biolabs, Inc.) (95˚ 3 min, 34 cycles of [95˚C 20 sec, 60˚C 1 min, 72˚C 1 min] 72˚C 5 min, 4˚C ∞) and gel imaging using GelRed Nucleic Acid Stain (Biotium, Fremont, CA, USA).

Target Genes Expression Analysis: RNA extraction and qPCR

RNA was extracted from 62 gill tissue samples from wild oysters collected in the summer of 2017 at Sister Lake A (n=15), Sister Lake B (n=15) Vermilion Bay (n=16), and Calcasieu Lake (n=16) Louisiana using either the GenElute™ Universal Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA) (n=61) or the E.N.Z.A. Total RNA Kit I (Omega Bio-tek, Inc.) (n=10). cDNA was made using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Thermocycler conditions: 25˚C 5min, 46˚C 20min, 95˚C 1 min, 4˚C forever). A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) was used to determine the concentration of RNA and dilutions were calculated for a beginning concentration of 3ng/µl for qPCRs.

To test for expression differences we used this cDNA from 62 wild oyster individuals and ran each gene in triplicate across two qPCR 96 well plates (31 individuals per plate) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and randomly run through either the Applied Biosystems™ ABI ViiA™ 7 or QuantStudio™ 6 Flex Real-Time PCR system (Thermo Fisher Scientific). Expression was normalized using the housekeeping gene myosin light chain kinase and expression differences across individuals and populations was calculated using the delta delta Ct ($2^{-\Delta\Delta ct}$) method (Schmittgen & Livak, 2008). We calculated z scores of each gene's $2^{-\Delta\Delta ct}$ values to assess variation in the expression of each gene among samples, each normalized to the scale of variation present in the expression of each gene. Heatmaps of z scores ($z = (x - \mu) / \sigma$) of $2^{-\Delta\Delta ct}$ were made for each target gene to look for

differences in expression across sampling locations and individuals. Kruskal-Wallis tests, a non-parametric test by ranks, were done for each gene across the three populations and two time points (4-way non-parametric ANOVAs).

We also performed a principle coordinate analysis (PCoA) by calculating Euclidean distances in the R package vegan to visualize clustering of the four genes sodium hydrogen exchanger, carnosine synthase, anti-apoptotic protein, and elongation factor 1-alpha by location and season (Figure 9). Significance was calculated using a permutational MANOVA using the adonis function in the R program vegan (2.5.2) (Anderson, 2001).

Results

Comparative Transcriptomics

Transcriptome Assembly and Read Mapping

Sequencing of the pooled libraries (24 total transcriptomes, 12 individuals in each of two lanes, and eight individuals in one lane (the 12 pools contained individuals for a different study)) resulted in an average of 34.1 ± 20.7 million reads per library in the two lanes with 12 individuals per lane and an average of 43.5 ± 10.9 reads in the single lane with eight individuals. These counts are from the FastQC files. After mapping to the publically available NCBI *C. virginica* transcriptome (GCA_002022765.4 C_virginica-3.0) we found one library that only had a mapping rate of 25.9%. After a Basic Local Alignment Search Tool (BLAST) query of the top overrepresented sequenced we determined that there were high levels of rRNA contamination in that sample and discarded it from further analysis (treatment group: WH). All treatment groups have four individuals except for WH which only has three. The other 23 transcriptomes aligned to the published transcriptome with a weighted average of 71.89% ± 1.6%. Of this total alignment percentage an average of 39.2% ± 3.15% aligned to multiple places in the transcriptome (the rest being unique alignments: average 31.8% ± 2.3%) .

In order to design primers specific to *C. virginica* in Louisiana we also assembled a *de novo* Trinity reference transcriptome. This assembly resulted in 33,081 total trinity genes, 54,559 total trinity transcripts, 40.89% GC content, N50 contig length 1,941, median contig length 1,079, average contig length 1,493.3, total assembled bases 81,472,726.

Differential Expression

Nine pairwise comparisons were done using a generalized linear model of the negative binomial family in edgeR (v3.20.7) and a total of 2,356 transcripts were identified to be differentially expressed with a FDR < 0.05. Number of up and down regulated transcripts for each comparison is shown in Figure 4. Within the three pairwise comparisons between the warm temperatures (WH vs. WL, WH vs. WM, WM vs. WL) there was an average of 400 (sd +/- 345) differentially expressed transcripts and within the three pairwise comparisons between the cool temperatures (CH vs. WL, CH vs. CM, CM vs. CL) there was an average of 180 (sd +/- 119) differentially expressed transcripts. This indicates a lot of variation between the different salinity treatments within the two temperatures.

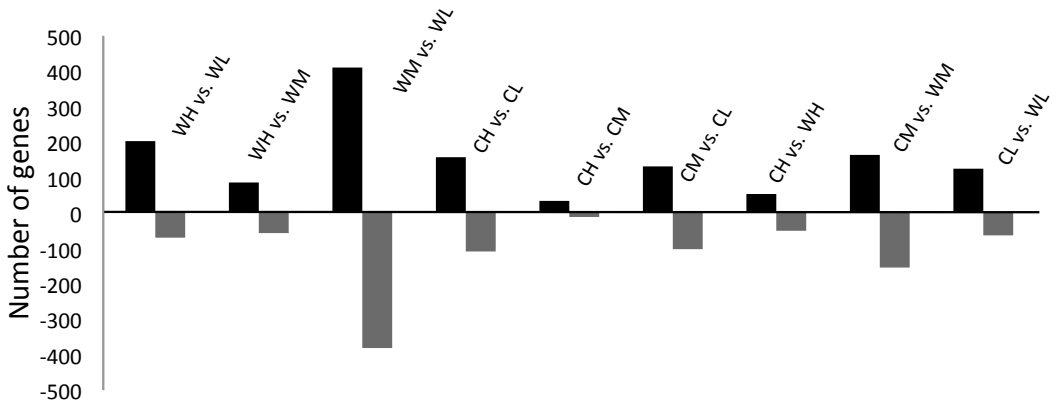

Figure 4. Differentially Expressed Gene Counts.
Number of upregulated (positive numbers) and downregulated (negative numbers) genes in each pairwise comparison made in our differential gene expression analysis in edgeR. Significantly differentially expressed by p value of 0.05 using the function decideTestsDGE in edgeR. These significantly differentially expressed genes undergo further trimming using a FDR of 0.05 so these numbers are slightly inflated.

Principle coordinate analysis of the differential gene expression estimated in edgeR revealed significant clustering by both temperature (adonis P < 0.05) and salinity (adonis P < 0.005) (Figure 4). Standard deviation ellipses in PCoA A revealed tight clustering of the low salinity and larger area for the medium and high salinities. PCoA B standard deviation ellipses show tighter clustering for the cooler temperature compared to the warmer temperature.

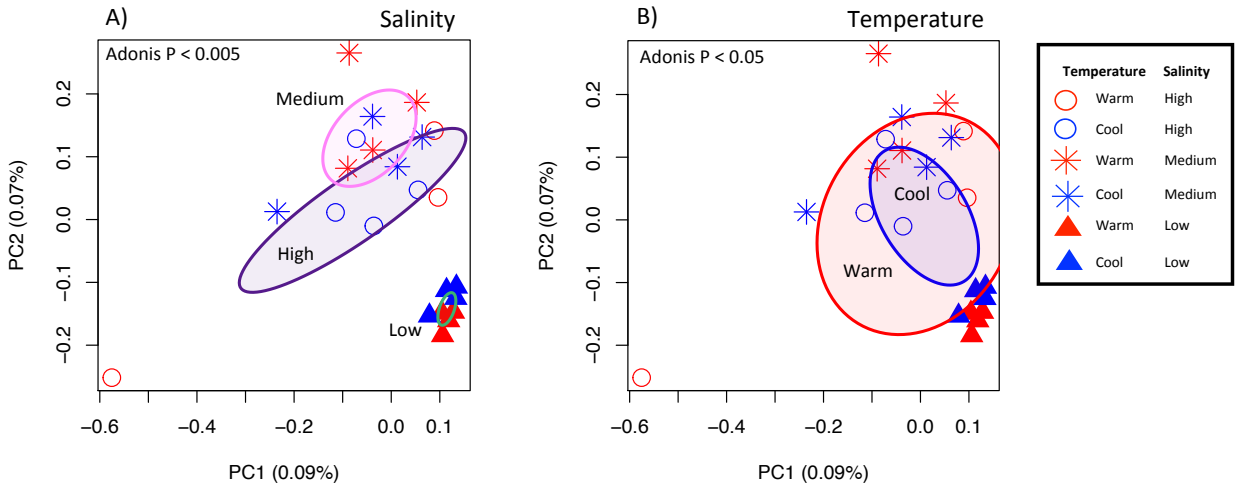

Figure 5. Principle Coordinate Analysis of Transcriptomes by Treatment.
Principal coordinate analysis using Euclidean distances of the 23 individual *C. virginica* samples' differential gene expression. A) Clustering of the three salinity levels (adonis P < 0.005). B) Clustering of the two temperature levels (adonis P < 0.05). Ellipses are standard deviations.

To look for differences between the number of differentially expressed transcripts in response to low salinity compared to medium salinity at the warm and cool temperatures, we plotted the log fold changes for the WM vs. WL and CM vs. CL pairwise comparisons (Figure 5). We found that the majority of transcripts had greater fold change in expression at the warmer temperature than the cooler temperature (732 out of 1027, Fisher's exact test, $p < 0.0001$).

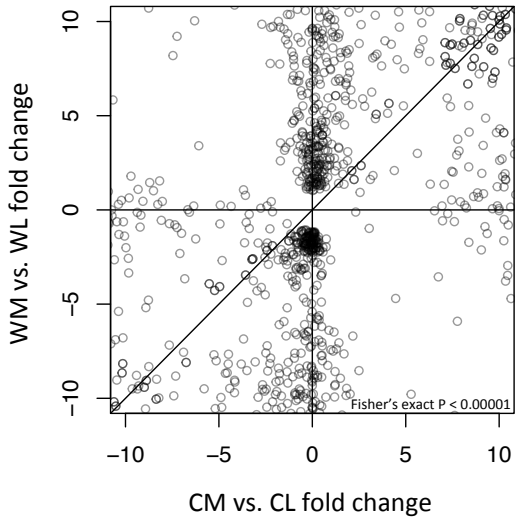

Figure 6. Log Fold Change of Genes Responding to Low Salinity at Warm Temperatures. Log fold change in expression for significantly differentially expressed transcripts in WM vs. WL and CM vs. CL pairwise comparisons. The one-to-one line divides upregulated transcripts with greater fold change in expression at the warmer temperature vs. the cooler temperature so that transcripts that were significantly upregulated fall above the line. We also counted transcripts that were more significantly downregulated in the warmer temperature so this includes points that fall below the one-to-one line but to the left of the center vertical line. The majority of transcripts had greater fold change in expression at the warmer temperature than the cooler temperature (732 out of 1027, Fisher's exact test, $p < 0.00001$).

Functional Enrichment

Functional enrichment analyses of the pairwise comparisons allowed us to look for general shifts in biological processes being differentially regulated in response to temperature and salinity stress. To represent changes in metabolism we chose 'cellular nitrogen compound metabolic process', 'oxoacid metabolic process', and 'regulation of metabolic process' GO terms which have 1215, 73, and 388 sequences respectively. The biological process GO term 'ion transport' (GO:0006811) is significantly enriched (adjusted $P < 0.05$) in two of our displayed comparisons in Table 2. Based on observations of significantly differentially regulated GO terms, as well as visual inspection of the MWU output files, we chose to highlight three terms that represented the metabolic response, four terms that represented the ion transport response, and five other terms that were significantly enriched in at least several different contrasts for a total of 12 GO terms. Table 2 shows these terms' responses in the WM vs. WL, CM vs. CL, and CM vs. WM comparisons. The first two comparisons are designed to show the low salinity response at the warm and cool temperatures while the third comparison is intended to represent

the warm temperature response at a normal salinity. In the WM vs. WL comparison we see upregulation up the four ion transport GO terms at the low salinity and in the CM vs. CL comparison we see a downregulation of the ion transport at the low salinity.

The GO term 'microtubule-based process' is upregulated at the low salinity treatment for both temperatures: WM vs. WL and CM vs. CL. The GO term 'G-protein coupled receptor signaling pathway' is significantly ($p < 0.05$ and $p < 0.00005$) downregulated at the low salinity treatment for both temperatures: WM vs. WL and CM vs. CL.

Table 2. Enriched Gene Ontology Terms.
GO terms with significantly differentially expressed genes from functional enrichment analysis for the following comparisons: WM vs. WL, CM vs. CL, and CM vs. WM. Shown are their regulation (+ for upregulated, - for downregulated), number of sequences expressed as a fraction, and adjusted p value for each comparison. If the term was not significantly expressed in one of the comparisons it is left blank. The regulation refers to the first treatment combination listed in the pairwise comparison. For example, in the first column, first row, the GO term 'cellular nitrogen compound metabolic process' is upregulated in the in WM treatment and downregulated in the WL treatment (represented by a +).

		WM vs. WL			CM vs. CL			CM vs. WM			
	GO term	regulation	nseqs	p adj	regulation	nseqs	p adj	regulation	nseqs	p adj	GO ID
Metabolic	cellular nitrogen compound metabolic process	+			-			-	447/1215	***	GO:0034641
	oxoacid metabolic process	-						-			GO:0019752;GO:0043436;GO:0006082
	regulation of metabolic process	+			+			-			GO:0031323;GO:0019222;GO:0051171;GO:0060255;GO:0080090;GO:0006355;GO:0010468;GO:1903506;GO:2000112;GO:2001141;GO:0010556;GO:0031326;GO:0051252;GO:0009889;GO:0019219
Ion Transport	exocytosis	-			-			-			GO:0006887;GO:1990504;GO:0017156;GO:0045055
	ion transport	-	137/324	**	+			+	131/324	*	GO:0006811
	potassium ion transport	-	27/56	*	+			+			GO:0006813
	transmembrane transport	-	188/463	**	+			+	177/463	***	GO:0055085
Other	G-protein coupled receptor signaling pathway	+	216/582	*	+	175/582	****	-			GO:0007186
	glycosaminoglycan biosynthetic process	-	11/17	*				+			GO:0006024;GO:0006023
	localization	-	324/792	*	-			+			GO:0006810;GO:0051234;GO:0051179
	microtubule-based process	-	31/67	*	-			+			GO:0007017
	signal transduction	+	405/1095	*	+	333/1095	***	-		**	GO:0007165

* $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$ **** $p < 0.00005$

Physiology

Respiration Rate

Respiration rate was calculated for 25 oysters from each treatment (total n =50) with a size range of 97.7 ± 13.2 mm in shell height and dry meat weights of 1.4 ± 0.8 g in the 30˚C treatment and 1.6 ± 0.8 g in the 20˚C treatment (individual shell heights and dry meat weights in A.1). Normality was assessed using the Shapiro-Wilk normality test ($p = 0.26$). There was a significant

effect of temperature on respiration rate with an increase from an average of 0.69 ± 0.22 mg O_2 h^{-1} at 20˚C to an average of 1.4 ± 0.33 mg O_2 h^{-1} at the warmer temperature of 30˚C (ANOVA p = 5.76e-12, F= 82.11) (see Figure 7a). Individual respiration rates are in A.1.

Clearance Rate

Clearance rate was calculated for 20 oysters from each treatment (total n = 40) with a size range of 98.7 ± 12.7 mm in shell height (individual shell heights in A.1) . Normality was assessed using the Shapiro-Wilk normality test and the data was transformed using the natural log to achieve normality (p = 0.07). There was no significant effect of temperature on the clearance rates with averages of 2.02 ± 1.3 L h^{-1} 100 mm^{-1} at 20˚C and 2.56 ± 1.64 L h^{-1}100 mm^{-1} at 30˚C (ANOVA p=0.356, F=0.871) (see Figure 7b). Individual clearance rates are in A.1.

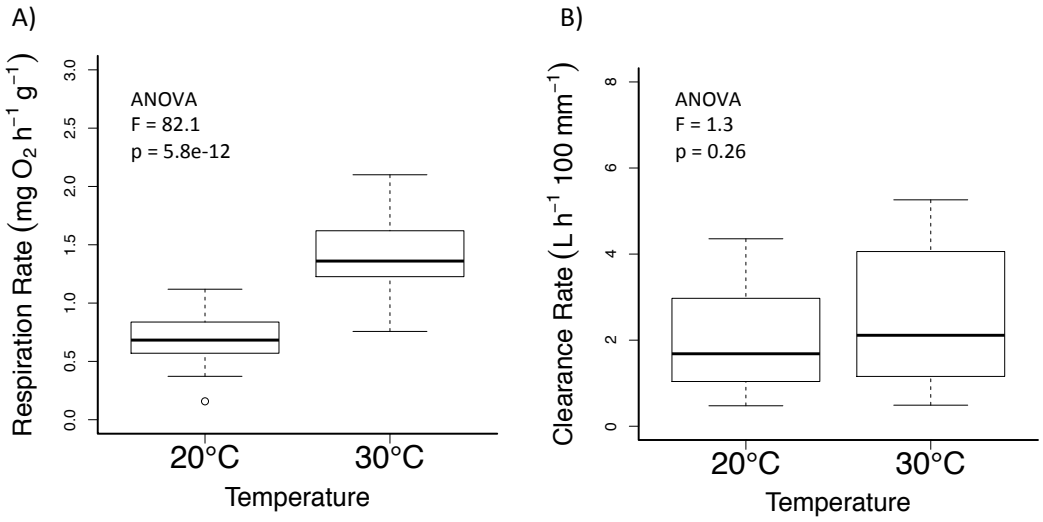

Figure 7. Box Plot of Respiration and Clearance Rate.
Respiration rate (mg O_2 h^{-1}) and clearance rate L h^{-1} 100 mm^{-1} for the two temperature treatments 20˚C and 30˚C at the single salinity of 7ppt. A) There was a significant effect of temperature on respiration rate with an increase from an average of 0.69 ± 0.22 mg O_2 h^{-1} at 20˚C to an average of 1.4 ± 0.33 mg O_2 h^{-1} at the warmer temperature of 30˚C (ANOVA p = 5.76e-12, $F_{1,48}$= 82.11). B) There was no significant effect of temperature on the clearance rates with averages of 2.02 ± 1.3 L h^{-1} 100 mm^{-1} at 20˚C and 2.56 ± 1.64 L h^{-1} at 30˚C (ANOVA p=0.356, $F_{1,38}$ =0.871).

Genes of Interest

Target Genes: Selection and Primer Design

Carnosine synthase, cadherin, and palmitoyltransferase were chosen as genes of interest to measure in field collected samples because of their differential expression in the comparative transcriptomic data. Their regulation is show in Table 3. Carnosine synthase was significantly upregulated in response to low salinity at the warm and cool temperatures (WM vs. WL and CM vs. CL), and at the low salinity at the cool temperature compared to the warm (WL vs. CL). It

was insignificantly upregulated in response to the warmer temperature across the medium salinity (CM vs. WM). Cadherin was significantly upregulated in response to low salinity across the cooler temperature (CM vs. CL) and at the low salinity at the cool temperature compared to the warm (WL vs. CL). Cadherin was insignificantly upregulated in response to low salinity at the warm temperature (WM vs. WL) and downregulated in response to the warm temperature across the medium salinity (CM vs. WM). Palmitoyltransferase was significantly upregulated in response to low salinity at the warm and cool temperatures (WM vs. WL and CM vs. CL), and at the low salinity at the cool temperature compared to the warm (WL vs. CL). It was not significantly upregulated in response to the warmer temperature across the medium salinity (CM vs. WM).

Table 3. Genes of Interest.
The seven putative biomarkers and the housekeeping gene (myosin light chain kinase) used in the qPCR analysis of field collected samples. Table includes gene name, accession number, forward position, product length, forward and reverse sequence, and the log fold change and p value associated with the WM vs. WL, WL vs. CL, CM vs. WM, and CM vs CL pairwise comparison made in our differential gene expression analysis in edgeR.

Gene Name	Accession Number	Forward Position	Product Length	Forward Primer Sequence (5' to 3') Reverse Primer Sequence (5' to 3')	WM_WL LFC	WM_WL PValue	WL_CL LFC	WL_CL PValue	CM_WM LFC	CM_WM Pvalue	CM_CL LFC	CM_CL PValue
Carnosine synthase	XM_022470031.1	801	175	CTACCCCGACACACTTGCTT CACAACAACCCGTTCGCATT	-	*	-	*	-		-	****
Elongation factor 1-alpha	XM_022472315.1	3001	177	GAGTGGAGATGCCGGTATGG GCCTTGGTCACTTTGCCTTG	+		-		-		-	
Heatshock protein 70	XM_022462566.1	2896	195	AAGATGCCGGAGTGATAGCG CAGTGGAGCGGACCTCAAAT	+		-		+		-	
Anti-apoptotic protein	XM_022477915.1	197	128	AAAATGAACCGGCAACTCGC TTCTGCCATCTGCCGAACTT	+		+		-		-	
Cadherin	XM_022480422.1	1639	150	GGCGGGTTGTTCTTTGTGAC AACAACAGCTACGTACGGGG	-		-	**	+		-	*
Palmitoyltransferase	XM_022467957.1	1581	196	GCCCAATGTAAGGGGGTTGA AGTTTGGGGTCATAGCACGG	+		-		-		-	
Sodium/hydrogen exchanger	XM_022459049.1	1781	127	CTCGCTCTGGATGCTGCTAT AAATGGCTATTGCCAGGGCT	-		-		+		-	
Myosin light chain kinase	XM_022490194.1	1123	118	CGTCGGCCAAATGGGATTTC CAGTCTCGTGCCGTCATTCT	+		+		-		+	

* $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$ **** $p < 0.00005$

Target Genes Expression Analysis: qPCR

The z scores of the $2^{-\Delta\Delta ct}$ qPCR results for the seven target genes were plotted as a heat map to compare transcript expression profiles (Figure 7). Particularly evident is the upregulation of EF1A, anti-apoptotic protein, carnosine synthase, and sodium hydrogen exchanger at the lower salinity location (Vermilion Bay), and the low salinity Sister Lake sampling time (B). Results from the Kruskal-Wallis tests indicated that each gene was differentially expressed in at least one of the four locations or time points (HSP70 P = 0.02, Palmityltransferase P = 0.02, Cadherin P = 1.1e-03, Sodium hydrogen exchanger P = 5.2e-06, Carnosine synthase P = 6.5e-04, Anti-apoptotic protein P = 4.0e-05, EF1A P = 4.3e-08).

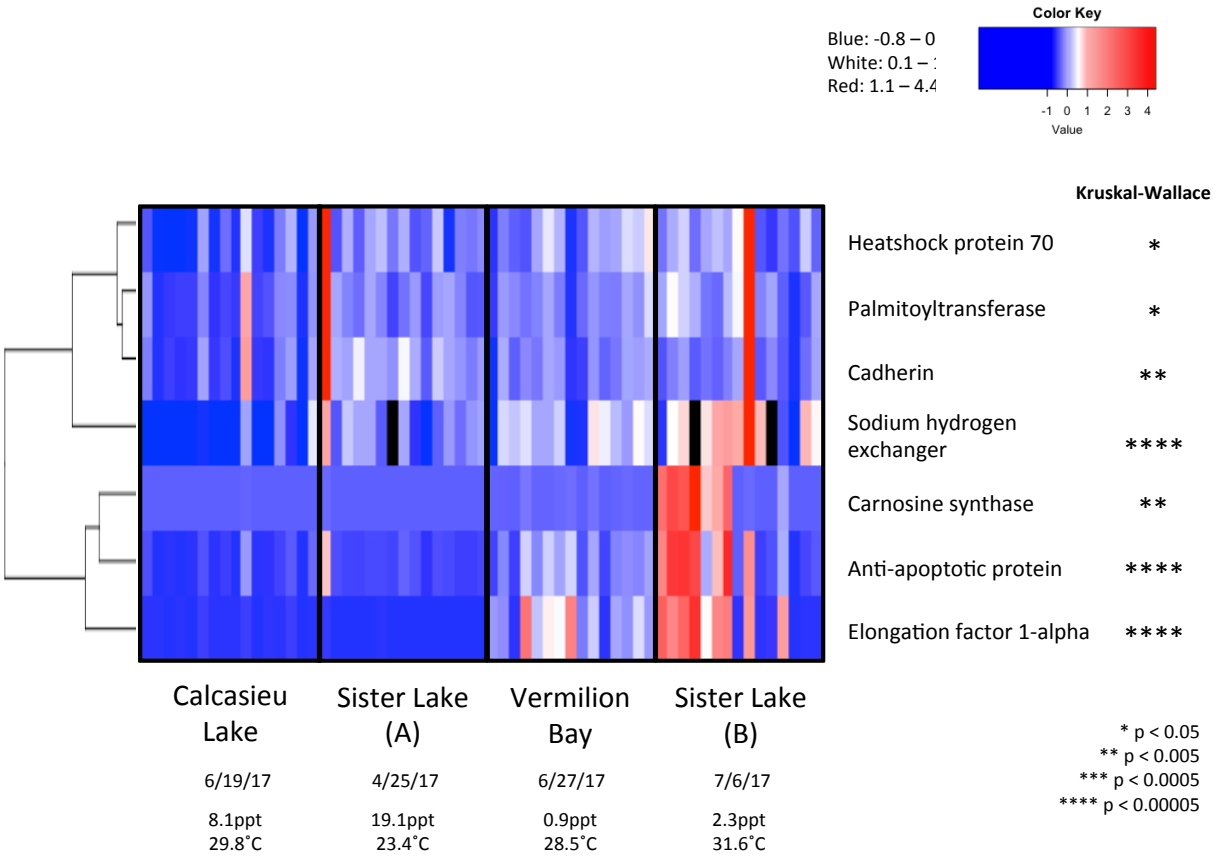

Figure 8. Heat Map of $2^{-\Delta\Delta}$ Values for Genes of Interest.
Heat map of z-scored $2^{-\Delta\Delta ct}$ values for putative biomarkers of combined temperature and salinity stress, standardized using the housekeeping gene myosin light chain kinase. Three locations and two time points shown: Calcasieu Lake (n=16), Vermilion Bay (n=16), Sister Lake A (n=15), Sister Lake B (n=15) with their collection date and the mean salinity and temperature on the day they were collected. The low salinity location is Vermilion Bay and the low salinity collection day is Sister Lake B. Black bars are N/As. Results from a 4-way Kruskal-Wallace non-parametric ANOVA show significant differences between the 4 sampling events for each gene.

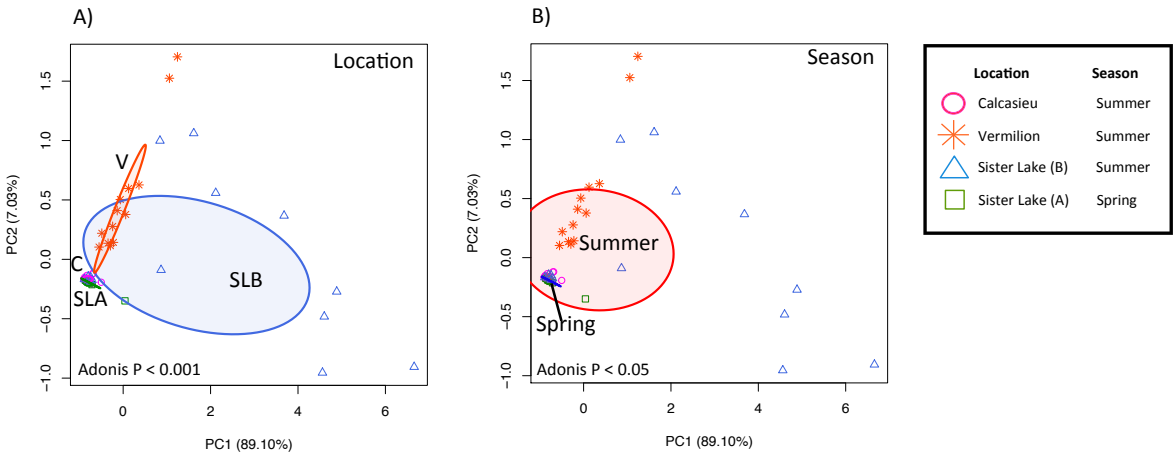

Figure 9. Principle Coordinate Analysis of $2^{-\Delta\Delta}$ Values by Location and Season.
Principle coordinates analysis using Euclidean distances of the $2^{-\Delta\Delta ct}$ for the four genes sodium hydrogen exchanger, carnosine synthase, anti-apoptotic protein, and elongation factor 1-alpha. Locations are indicated by labels (SLA for the Spring Sister Lake sampling date, SLB for the Summer Sister Lake sampling date, C for Calcasieu Lake, and V for Vermilion Bay). Ellipses are standard deviations. A) PCoA A ellipses show the clustering of the four locations (adonis $P < 0.001$). B) PCoA B ellipse show the clustering of the two seasons (adonis $P < 0.05$).

Discussion

Our study examines the effects of combined temperature and salinity stress (two important and regularly fluctuating stressors in the coastal marine environment) on the transcriptomic and physiological responses in the eastern oyster, *C. virginica*. Using three different approaches, we found that temperature and salinity have synergistic, non-additive effects on *C. virginica*, meaning that the effects of multiple stressors was greater than the sum of their individual effects (Rachel Przeslawski, Byrne, & Mellin, 2015). When *C. virginica* was exposed to low salinities (7ppt) the magnitude of their phenotypic response, in terms of number of genes differentially expressed, was much greater at warmer temperatures (30˚C) than cooler temperatures (20˚C) (Figure 4 & 6). Looking at PCoAs of their changes in gene expression (Figure 5) we also see that the oysters exposed to the low salinities are having a much more similar response (tighter ellipse of standard deviation) than the oysters at the medium and high salinities, which are showing more variation in their response. Functional enrichment analysis revealed that the GO terms relating to metabolism and ion transport were significantly enriched, indicated that metabolism and ion transport (homeostasis) play major roles in the molecular response to combined temperature and salinity stress (Table 2).

C. virginica clearance rate did not significantly increase at the warmer temperature like respiration rate did (Figure 7), indicating an energetic gap. Casas et al (2018), documented winter (17˚C) and summer (27˚C) clearance and respiration rates for Louisianan oysters at 2, 6, 9, 15, and 25 ppt and found that salinity as well as seasonality (reproductive stage) affected oyster physiology. They found reduced feeding rates in the summer oysters at the low salinities with a threshold between 6 and 9 ppt where rates decreased; at the lowest salinity of 3ppt both summer and winter clearance rates were severely depressed. Clearance rates can be used to estimate the amount of energy ingested over time (Casas, Lavaud, et al., 2018) so we can assume that while we didn't see a reduction in clearance rate for our oysters at 7ppt between our two temperature treatments of 20˚C and 30˚C that there might be a threshold below 6ppt when they are not able to create as much energy because of reduced feeding rates. This reduction of energy intake coupled with the increased energetic demands of the hypo-osmotic stress response can lead to increased mortality in oysters.

Casas et al (2018) also observed that oyster respiration rates were four times higher in the summer than the winter and that winter respiration was statistically the same across all five salinities but varied between salinities during the summer and attributed this to different stages of gonadal development. We also observed an approximately two times higher respiration rate at 30˚C compared to 20˚C at 7ppt salinity. Differences could be due to the fact that our oysters were larger (with shell heights about 30 mm longer on average) or because of disparity in gonadal development stage. Respiration rate can be used to estimate metabolic costs (Casas, Lavaud, et al., 2018) and is important when considering energetic tradeoffs of combined temperature and salinity stress.

We saw significant differences in expression of our seven target genes in our qPCR analysis across Calcasieu Lake, Vermilion Bay, and the two time points at Sister Lake. Surprisingly, we found that EF1A, a protein synthesis gene typically considered a housekeeping gene, showed significant upregulation in Vermilion Bay and the Sister Lake oysters collected in the summer (Figure 7). Housekeeping genes are generally constitutively expressed genes involved in basic cellular maintenance and do not change expression levels in different environmental conditions so we propose that EF1A is not reliable for this purpose. Respectively,

the salinities at the time of sampling and the 2017 average salinity for each site were: Calcasieu Lake (8.1ppt, 19.8ppt), Vermilion Bay (0.9ppt, 4.2ppt), Sister Lake A (19.1ppt, 12.2ppt), and Sister Lake B (2.3ppt, 12.2ppt). Both the Vermilion Bay and Sister Lake B sampling events took place during salinity exposure below 7ppt which is why we might have seen such high expression of EF1A that was not reflected in the transcriptomic data.

Sister Lake (B) appears to have the highest level of upregulation across the seven target genes indicating the most extreme response. Looking back at Table 1, Sister Lake experiences an annual average salinity of 12.2 ± 5.4ppt and was experiencing 5.0 ± 2.6 ppt for the month prior to sampling. Vermilion Bay had the lowest salinity for the month prior to sampling (0.8 ± 0.3 ppt) but experiences an annual average of 4.2 ± 3.1ppt. Calcasieu Lake experienced a mild 11.4 ± 4.1ppt for the month leading up to sampling possibly explaining its generally lower response across the seven target genes.

Synergistic Effects of Temperature and Salinity Stress

In our differential expression analyses we saw low overlap between responses in the warm and cool temperatures at different salinities, indicating that the responses to each individual stressor is a result of a different response pathway. Modulation of energy metabolism is a key player in the cellular stress response (Kültz, 2005) and the energetic demands resulting from maintaining both pathways can result in energetic tradeoffs. For example, oysters show little evidence for suppression of metabolic energy demands in the summer and the combination of temperature and reproduction can result in a fourfold oxygen demand in the summer compared to the winter (Casas, Lavaud, et al., 2018).

Additionally, in the summer is when we see the lowest salinities at our sites because of increased rainfall and outflow from the Mississippi and Atchafalaya Rivers. When oysters are faced with low or high salinities they need to A) regulate inorganic and organic osmolytes to maintain cell volume (at a metabolic cost) or at extremely low salinities (below 5 – 3ppt) B) close their valves to the environment which halts feeding and results in anaerobic metabolism (Lavaud, La Peyre, Casas, Bacher, & La Peyre, 2017). Because oysters lack the ability to osmoregulate their extracellular fluid, the regulation of cell volume in response to changing salinity through the regulation of organic and inorganic osmolytes can be energetically costly. The use of organic osmolytes (taurine, alanine, glycine, etc) is much less costly than inorganic osmolytes (N+, K+, Ca2+) (Eierman & Hare, 2014). Additionally, cell membranes are more permeable during periods of increased temperature stress, which will change for different species and populations but for our Louisianan oysters may be around 30˚C, and this increased permeability can decrease the efficacy of active transport of inorganic osmolytes (Kelly, DeBiasse, et al., 2016). Oysters also may be changing membrane lipid composition in response to higher temperatures (Somero, 2002). It has been observed that at salinities of 3 or below *C. virginica* will remain closed to isolate themselves from the low salinity and there is elevated death and gonad reabsorption because of the higher energetic demands of higher temperatures (such as 30˚C) and the lack feeding/costs of anaerobic metabolism (Casas, Lavaud, et al., 2018).

All four hypothesized ion transport GO terms (‘exocytosis’, ‘ion transport’, ‘potassium ion transport’, and ‘transmembrane transport’) were upregulated in response to low salinity at 30˚C (WM vs. WL) while three of the four (excluding ‘exocytosis’) were downregulated in response to low salinity at 20˚C (CM vs. CL). Lockwood & Somero (2011) described the transcriptomic response to salinity stress in two *Mytilus* species, also marine bivalve

osmoconformers, and compared this response to a 2010 study they both participated in that characterized the transcriptomic response of these two mussel species to heat stress (B. L. Lockwood, Sanders, & Somero, 2010; Brent L. Lockwood & Somero, 2011). They found that of the 45 genes that were differentially expressed in response to both stressors, the genes most strongly upregulated by heat stress were most strongly downregulated by hypo-osmotic stress. The GO term 'ion transmembrane transporter activity' was significantly over-represented in the 45 genes. This may be because upregulation of ion channels allows ions to move freely across the cell membrane to equalize concentrations on either side, but downregulation allows ion channels to stop the transport of solutes into the cytosol where the solute concentration is already too high. They postulate that the effect that heat stress has on lipid membrane permeability (Kültz, 2005) may also be playing a role in the regulation of these genes.

The GO term 'microtubule-based process' was upregulated in the low salinity treatment for both 20˚C and 30˚C. We hypothesize that this is because of increased ciliary activity that can improve the ability to transport fluids within the mantle cavity which can prolong aerobic metabolism and survival at extremely low salinities when oysters close their valves (Maynard, Bible, Pespeni, Sanford, & Evans, 2018).

The GO term 'G-protein coupled receptor signaling pathway' was significantly downregulated in response to low salinity at the warm and cool temperatures (WM vs. WL and CM vs. CL) (Table 2). This GO term is a 'child' term of 'hydrolase activity' which is involved in phosphorylation of the plasma membrane proteins and hydrolysis of peptides which both create organic osmolytes in order to maintain homeostasis (Eierman & Hare, 2014). Studying the expression of genes that move these inorganic and organic osmolytes across cell membranes, such as taurine transporters, could be useful in future stress studies (Eierman & Hare, 2014; J. Meng et al., 2013).

Warmer temperatures increase oxygen demand until a threshold temperature where the organism cannot take up enough oxygen to meet demands – resulting in anaerobic metabolism and energetic failure. Casas et al. (2018) found a significant increase in clearance rate of Louisiana oysters from 10˚C to 20˚C and 30˚C (but not between 20˚C and 30˚C) and a significant increase in oxygen consumption at higher temperatures (Casas, Lavaud, et al., 2018). Oysters may be experiencing an unknown 'breaking point' above 30˚C for oxygen consumption. Identifying temperature and salinity turning points for different populations should be considered in future studies; defining thresholds could help us identify especially vulnerable populations (Evans & Hofmann, 2012).

Contrasting Laboratory and Wild Expression

Gene expression measured in a laboratory setting will be different from expression in the field because organisms in natural habitats experience fluctuations in multiple factors that may not vary in laboratory environments. This could be for a multitude of reasons, including variation in environmental stressors that we didn't consider. The salinities at Vermilion Bay and the summer Sister Lake sampling time point were lower than the salinities the oysters were exposed to in the lab. Laboratory oysters were also held at constant salinities for three weeks before tissue sampling while the Sister Lake B oysters were sampled two weeks after the large influx of freshwater brought to the GOM by Tropical Storm Cindy (See Figure 3A for 2017 daily mean salinities).

The dramatic upregulation of EF1A, typically considered a housekeeping gene, in the low salinity population (Vermilion Bay) and low salinity sampling date (Sister Lake B) is not reflected in the laboratory exposure. In an evaluation of qPCR reference genes used in climate change stress studies, Shimpi et al. (2016) found EF1A to be a highly variable housekeeping gene in the octocoral *Sinularia cf. cruciata* during low–pH and thermal stress (Shimpi, Vargas, & Wörheide, 2016). EF1A has also been shown to play a part in the production of proteins associated with the heat shock response (Buckley, Andrew, & George, 2006; Wares & Schiebelhut, 2016).

We also saw significant differential expression in palmitoyltransferase and cadherin in the transcriptomic results (Table 3) and more heterogeneous expression across populations in the qPCR results (Figure 7) where the significant differences are mostly driven by two individual outliers. We were surprised by the lack signal observed in HSP70 expression (Sørensen, Kristensen, & Loeschcke, 2003) but, predict that it could be due to the pattern observed in Meyer et al. (2011) where HSPs were upregulated in the short term temperature stress treatments but, down-regulated or constant in long-term exposure (Meyer, Aglyamova, & Matz, 2011). Further sampling of wild populations should be done to determine what these genes are actually signaling and whether they have potential as biomarkers of combined temperature and salinity stress.

Chaney & Gracey (2011) used biomarkers to predict *Crassostrea gigas* mass mortality events. They used nonlethal methods of sampling that could be applied to *C. virginica* during the summer when there is elevated mortality because of the combination of environmental stressors and the increased demands of gametogenesis and spawning to quantify the expression of stress response genes (Chaney & Gracey, 2011). This could be used to identify populations more resistant to summer mortality (at the cost of slower gametogenesis and less reproductive effort) (Huvet et al., 2010). This resistance to mortality is a highly heritable trait (Sauvage et al., 2010) and should be considered when identifying resilient populations.

Genes differentially expressed under stress are more likely to have paralogues, so it can be even more difficult to capture the stress response using qPCR methodology. Zhang et al. (2012) found 88 heat shock protein 70 genes in the Pacific oyster, *Crassostrea gigas*, which respond to a multitude of stressors (Zhang et al., 2012). Long-term versus short-term exposure to stress can also result in important differences in the stress response (Guzman & Conaco, 2016; Meyer et al., 2011). Different expression pathways, linkage, or pleiotropy may also be involved in expressing the same gene, such as HSP90, which can be transcribed for a multitude of reasons (Louis, Bhagooli, Kenkel, Baker, & Dyall, 2017; Schoville et al., 2012).

Future Directions

The literature on the effects of climate change on marine organisms has mostly focused on single stressors and almost 60% of these publications have looked at solely temperature (Harley et al., 2006). It is important that any future work consider the complex effects of combined environmental stressors in order to not drastically underestimate its ecological impacts.

When predicting the effects of climate change on eastern oyster populations it will also be important to consider their historical exposure. Leonhardt et al (2017) collected oysters from three Louisiana estuaries (Sister Lake, Lake Calcasieu, and Breton Sound) and deployed their progeny across a salinity gradient in coastal Louisiana. Progeny from Sister Lake consistently

showed the lowest mortality during intervals of low salinity potentially due to their year round intermediate to low salinity regime and possible adaptation (Leonhardt et al., 2017).

Louisiana's Comprehensive Master Plan for a Sustainable Coast proposes sediment and freshwater diversions, both of which would introduce freshwater from the Mississippi River into estuarine environments (CPRA, 2017). It is important that we consider the seasonality of these diversions so that they do not coincide with warm temperatures or during spawning events. Oysters spawn twice a year (late spring and early fall) with 25˚C as the critical temperature for stimulating gamete production (LDWF, 2016). Timing sediment and freshwater diversions to account for oyster can improve recruitment and survival.

There is a need for the ability to assess oyster condition in the field after a stressful event like a hurricane or freshwater diversion. When we look at the combined expression of the four genes (sodium hydrogen exchanger, carnosine synthase, anti-apoptotic protein, and EF1A) used to make the PCoA of qPCR expression (Figure 9) we can see a break along PC1 at around 0 where the low salinity sampling (Vermilion Bay and Sister Lake in the Summer) separate out from the high salinity sampling (Calcasieu Lake and Sister Lake in the Spring). Ideally, if we were given an oyster from unknown conditions, and did the same analysis using these four genes we would be able to classify how it is coping with combined temperature and hypo-osmotic stress. The ability to quickly classify oyster condition using gene expression would be useful for monitoring efforts but at this point in time requires testing more locations, environmental conditions, and genes of interest.

Limitations and Considerations

Because of the time sensitive nature of transcriptomics we acknowledge that comparisons made between individuals exposed in different years needs to be taken with a grain of salt, despite the application of identical methods.

The transcriptomic response is necessarily dynamic and can rapidly be adjusted to react to environmental changes. This can lead to challenges and complications when utilizing RNA-seq. Transcriptional differences are extremely variable between individuals, tissue specific (Leung et al., 2014), and time specific. Discrepancies between library preparation batches, commercial sequencers, and bioinformatics approaches can also result in downstream bias (Gleason & Burton, 2015; Hofmann & Place, 2007; Ozsolak & Milos, 2011). Additionally, the presence of a transcript does not necessarily mean that a protein resulted and post-transcriptional and allosteric modifications can only be observed through proteomic analysis (Louis et al., 2017). Epigenetics may also be the cause of some of the differential expression seen between populations and it is suggested that more experiments be done in concert with epigenetics analysis (Seneca & Palumbi, 2015).

Conclusions

We tested both physiological and genetic trade-offs in *C. virginica* in response to two important environmental stressors, temperature and salinity, using transcriptome data, physiology experiments, and quantitative real-time PCR At high temperatures, oysters response significantly to low salinities, as reflected in their transcriptome with increase expression of genes that control ion transport and ciliary action in response to low salinity. The timing and duration of freshwater events will have large impacts of eastern oyster recruitment and survival as temperatures

gradually warm and push them closer to their tolerance thresholds. Linking environment, physiology, and gene expression profiles can help us create a more complete picture of how climate change will impact phenotypically plastic organisms (J. Meng et al., 2013). The cascading and sometimes unpredictable impacts of multiple stressors is why studies that measure responses to a single climatic stressor will be inadequate when predicting the effects of future environmental change (R. Przeslawski et al., 2005).

Appendix: Oyster Measurements

Table A.1. Oyster Shell Height, Dry Meat Weight, and Individual Physiological Measurements. Individual oyster height (mm), dry meat weight (g), respiration rate (mg O2 h^{-1} g^{-1}), and clearance rate (L h^{-1} $100mm^{-1}$) for the 2017 oysters used for physiological measurements.

Temperature treatment (˚C)	Height (mm)	Dry meat (g)	Respiration rate (mg O2 h^{-1} g^{-1})	Clearance rate (L h^{-1} $100mm^{-1}$)
20	95.8	2.05	0.892	
20	105.8	1.25	0.649	
20	82.8	1.11	0.480	2.375
20	98	3.27		1.158
20	95	1.88	0.655	1.525
20	107.9	2.53		0.499
20	98.4	1.67	0.789	0.921
20	91.6	1.81	0.852	0.581
20	106.7	3.33	0.521	4.356
20	74	0.34	0.406	
20	67.7	0.21		1.838
20	75.7	0.75	0.837	4.209
20	83.7	0.52	0.372	
20	92.8	1.94	0.856	
20	89.4	2	0.736	
20	101.6	1.36	0.748	1.773
20	77.5	0.86	0.158	1.638
20	112.3	1.73	0.685	
20	79.4	0.63	0.570	
20	112	1.72	1.046	0.475
20	99.5	1.27	0.946	1.549
20	99.8	1.36	0.678	3.700
20	105.2	3.08	0.683	2.360
20	106.1	1.83	0.789	1.729
20	106.4	1.58	0.596	4.087
20	88.4	1.31	0.617	
20	72.3	0.62		
20	95.1	1.12		1.163
20	76.5	0.61		3.570
20	117.2	2.78	0.497	
20	95.5	0.89	1.119	
20	113.3	3.72		0.920
30	95.5	0.94	1.620	0.489
30	100	1.54	1.357	2.067
30	94	1.07	1.117	
30	96	1.43	1.840	1.239
30	119.5	1.48	1.632	0.917
30	80.8	0.56	1.584	
30	79.4	0.54	0.760	1.531
30	99.9	2.67		4.963
30	97.4	1.29	1.414	
30	80	0.77	1.060	
30	111.6	1.36		2.893
30	111.7	2.78	1.437	1.076
30	113.4	1.75		2.160
30	119.1	0.87		1.282
30	109.4	1.09	1.688	

30	96.6	1.74	1.237	5.096
30	124.5	3.54	1.068	
30	110.9	0.85	1.290	
30	102.3	1.37		3.657
30	110.6	3.39	0.757	0.954
30	111.4	1.77	1.349	4.461
30	91.1	1.47	1.227	1.887
30	84.7	0.36	1.509	
30	85.2	0.76	2.100	2.874
30	100.4	1.67	1.360	4.844
30	108.3	2.11	1.357	5.261
30	108	1.13	1.691	
30	98.2	1	1.922	
30	105.1	1.31	1.532	0.552
30	85.8	1.51	1.094	2.930

Literature Cited

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), 32–46. doi:10.1046/j.1442-9993.2001.01070.x

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Biasutti, M., Sobel, A. H., Camargo, S. J., & Creyts, T. T. (2012). Projected changes in the physical climate of the Gulf Coast and Caribbean. *Climatic Change*, *112*(3–4), 819–845. doi:10.1007/s10584-011-0254-y

Blunden, J., Arndt, D. S., Blunden, J., & Arndt, D. S. (2016). State of the Climate in 2015. *Bulletin of the American Meteorological Society*, *97*(8), Si-S275. doi:10.1175/2016BAMSStateoftheClimate.1

Boldyrev, A. A., Aldini, G., & Derave, W. (2013). Physiology and Pathophysiology of Carnosine. *Physiological Reviews*, *93*(4), 1803–1845. doi:10.1152/physrev.00039.2012

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. doi:10.1093/bioinformatics/btu170

Buckley, B. A., Andrew, G., & George, S. (2006). The cellular response to heat stress in the goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. *Journal of Experimental Biology*, *209*(14), 2660–2677. doi:10.1242/jeb.02292

Casas, S. M., Filgueira, R., Lavaud, R., Comeau, L. A., La Peyre, M. K., & La Peyre, J. F. (2018). Combined effects of temperature and salinity on the physiology of two geographically-distant eastern oyster populations. *Journal of Experimental Marine Biology and Ecology*, *506*(June), 82–90. doi:10.1016/j.jembe.2018.06.001

Casas, S. M., Lavaud, R., La Peyre, M. K., Comeau, L. A., Filgueira, R., & La Peyre, J. F. (2018). Quantifying salinity and season effects on eastern oyster clearance and oxygen consumption rates. *Marine Biology*, *165*(5), 1–13. doi:10.1007/s00227-018-3351-x

Chaney, M. L., & Gracey, A. Y. (2011). Mass mortality in Pacific oysters is associated with a specific gene expression signature. *Molecular Ecology*, *20*(14), 2942–2954. doi:10.1111/j.1365-294X.2011.05152.x

Chapman, R. W., Mancia, A., Beal, M., Veloso, A., Rathburn, C., Blair, A., … Sanger, D. (2011). The transcriptomic responses of the eastern oyster, Crassostrea virginica, to environmental conditions. *Molecular Ecology*, *20*(7), 1431–1449. doi:10.1111/j.1365-294X.2011.05018.x

DeBiasse, M. B., & Kelly, M. W. (2016). Plastic and evolved responses to global change: What can we learn from comparative transcriptomics? *Journal of Heredity*, *107*(1), 71–81. doi:10.1093/jhered/esv073

Dickinson, G. H., Ivanina, A. V, Matoo, O. B., Pörtner, H. O., Lannig, G., Bock, C., … Sokolova, I. M. (2012). Interactive effects of salinity and elevated CO2 levels on juvenile eastern oysters, Crassostrea virginica. *The Journal of Experimental Biology*, *215*, 29–43. doi:10.1242/jeb.061481

Du, Y., Zhang, L., Xu, F., Huang, B., Zhang, G., & Li, L. (2013). Validation of housekeeping genes as internal controls for studying gene expression during Pacific oyster (Crassostrea gigas) development by quantitative real-time PCR. *Fish & Shellfish Immunology*, *34*(3), 939–945. doi:10.1016/j.fsi.2012.12.007

Eierman, L. E., & Hare, M. P. (2014). Transcriptomic analysis of candidate osmoregulatory genes in the eastern oyster Crassostrea virginica. *BMC Genomics*, *15*(1). doi:10.1186/1471-2164-15-503

Evans, T. G., & Hofmann, G. E. (2012). Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1596), 1733–1745. doi:10.1098/rstb.2012.0019

Gardner, J. P. A., & Thompson, R. J. (2001). The effects of coastal and estuarine conditions on the physiology and survivorship of the mussels Mytilus edulis, M-trossulus and their hybrids. *Journal of Experimental Marine Biology and Ecology*, *265*(2), 119–140. doi:Doi 10.1016/S0022-0981(01)00328-8

George, L. M., De Santiago, K., Palmer, T. A., & Beseres Pollack, J. (2015). Oyster reef restoration: effect of alternative substrates on oyster recruitment and nekton habitat use. *Journal of Coastal Conservation*, *19*(1), 13–22. doi:10.1007/s11852-014-0351-y

Gleason, L. U., & Burton, R. S. (2015). RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail Chlorostoma funebralis. *Molecular Ecology*, *24*(3), 610–627. doi:10.1111/mec.13047

Gómez-Chiarri, M., Warren, W. C., Guo, X., & Proestou, D. (2015). Developing tools for the study of molluscan immunity: The sequencing of the genome of the eastern oyster, Crassostrea virginica. *Fish & Shellfish Immunology*, *46*(1), 2–4. doi:10.1016/j.fsi.2015.05.004

Grabowski, J. H., Brumbaugh, R. D., Conrad, R. F., Keeler, A. G., Opaluch, J. J., Peterson, C. H., … Smyth, A. R. (2012). Economic Valuation of Ecosystem Services Provided by Oyster Reefs. *BioScience*, *62*(10), 900–909. doi:10.1525/bio.2012.62.10.10

Guzman, C., & Conaco, C. (2016). Gene expression dynamics accompanying the sponge thermal stress response. *PLoS ONE*, *11*(10), 1–15. doi:10.1371/journal.pone.0165368

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Philip, D., Bowden, J., … Pochet, N. (2014). *De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis with Trinity*. *Nat Protocols* (Vol. 8). doi:/10.1038/nprot.2013.084.De

Harley, C. D. G., Hughes, A. R., Hultgren, K. M., Miner, B. G., Sorte, C. J. B., Thornber, C. S., … Williams, S. L. (2006). The impacts of climate change in coastal marine systems. *Ecology Letters*, *9*(2), 228–241. doi:10.1111/j.1461-0248.2005.00871.x

Heilmayer, O., Digialleonardo, J., Qian, L., & Roesijadi, G. (2008). Stress tolerance of a subtropical Crassostrea virginica population to the combined effects of temperature and salinity. *Estuarine, Coastal and Shelf Science*, *79*(1), 179–185. doi:10.1016/j.ecss.2008.03.022

Hofmann, G. E., & Place, S. P. (2007). Genomics-enabled research in marine ecology: Challenges, risks and pay-offs. *Marine Ecology Progress Series*, *332*(1), 249–255. doi:10.3354/meps332249

Huth, T. J., & Place, S. P. (2016). Transcriptome wide analyses reveal a sustained cellular stress response in the gill tissue of Trematomus bernacchii after acclimation to multiple stressors. *BMC Genomics*, 1–18. doi:10.1186/s12864-016-2454-3

Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., & Boudry, P. (2010). Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality. *Aquaculture*, *304*(1–4), 95–99. doi:10.1016/j.aquaculture.2010.03.022

Joshi, M. B., Philippova, M., Ivanov, D., Allenspach, R., Erne, P., & Resink, T. J. (2005). T-cadherin protects endothelial cells from oxidative stress-induced apoptosis. *The FASEB Journal*, *19*(12), 1737–1739. doi:10.1096/fj.05-3834fje

Kelly, M. W., DeBiasse, M. B., Villela, V., Roberts, H., & Cecola, C. (2016). Adaptation to climate change: trade-offs among responses to multiple stressors in an intertidal crustacean. *Evolutionary Applications*, 1–9. doi:10.1111/eva.12394

Kelly, M. W., Pankey, M. S., Debiasse, M. B., & Plachetzki, D. C. (2016). Adaptation to heat stress reduces phenotypic and transcriptional plasticity in a marine copepod. *Functional Ecology*, 398–406. doi:10.1111/1365-2435.12725

Kültz, D. (2005). Molecular and Evolutionary Basis of the Cellular Stress Response. *Annual Review of Physiology*, *67*(1), 225–257. doi:10.1146/annurev.physiol.67.040403.103635

La Peyre, M. K., Eberline, B. S., Soniat, T. M., & La Peyre, J. F. (2013). Differences in extreme low salinity timing and duration differentially affect eastern oyster (Crassostrea virginica) size class growth and mortality in Breton Sound, LA. *Estuarine, Coastal and Shelf Science*, *135*, 146–157. doi:10.1016/j.ecss.2013.10.001

La Peyre, M. K., Nickens, A. D., Volety, A. K., Tolley, G. S., & La Peyre, J. F. (2003). Environmental significance of freshets in reducing Perkinsus marinus infection in eastern oysters Crassostrea virginica: Potential management applications. *Marine Ecology Progress Series*, *248*, 165–176. doi:10.3354/meps248165

Laing, I. (2002). Effect of salinity on growth and survival of king scallop spat (*Pecten maximus*). *Aquaculture*, *205*(1–2), 171–181. doi:10.1016/S0044-8486(01)00663-9

Lancaster, L. T., Dudaniec, R. Y., Chauhan, P., Wellenreuther, M., Svensson, E. I., & Hansson, B. (2016). Gene expression under thermal stress varies across a geographical range expansion front. *Molecular Ecology*, *25*(5), 1141–1156. doi:10.1111/mec.13548

Lauritano, C., Procaccini, G., & Ianora, A. (2012). Gene expression patterns and stress response in marine copepods. *Marine Environmental Research*, *76*, 22–31. doi:10.1016/j.marenvres.2011.09.015

Lavaud, R., La Peyre, M. K., Casas, S. M., Bacher, C., & La Peyre, J. F. (2017). Integrating the effects of salinity on the physiology of the eastern oyster, Crassostrea virginica, in the northern Gulf of Mexico through a Dynamic Energy Budget model. *Ecological Modelling*, *363*, 221–233. doi:10.1016/j.ecolmodel.2017.09.003

Leonhardt, J. M., Casas, S., Supan, J. E., & La Peyre, J. F. (2017). Stock assessment for eastern oyster seed production and field grow-out in Louisiana. *Aquaculture*, *466*, 9–19. doi:10.1016/j.aquaculture.2016.09.034

Leung, P. T., Ip, J. C., Mak, S. S., Qiu, J., Lam, P. K., Wong, C. K., … Leung, K. M. (2014). De novo transcriptome analysis of Perna viridis highlights tissue-specific patterns for environmental studies. *BMC Genomics*, *15*(1), 804. doi:10.1186/1471-2164-15-804

Li, A., Li, L., Song, K., Wang, W., & Zhang, G. (2017). Temperature , energy metabolism , and adaptive divergence in two oyster subspecies, (April), 1–12. doi:10.1002/ece3.3085

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, *12*. doi:10.1186/1471-2105-12-323

Li, Y., Qin, J. G., Abbott, C. A., Li, X., & Benkendorff, K. (2007). Synergistic impacts of heat shock and spawning on the physiology and immune health of Crassostrea gigas: an explanation for summer mortality in Pacific oysters. *AJP: Regulatory, Integrative and Comparative Physiology*, *293*(6), R2353–R2362. doi:10.1152/ajpregu.00463.2007

Lockwood, B. L., Sanders, J. G., & Somero, G. N. (2010). Transcriptomic responses to heat stress in invasive and native blue mussels (genus Mytilus): molecular correlates of invasive success. *Journal of Experimental Biology*, *213*(20), 3548–3558. doi:10.1242/jeb.046094

Lockwood, B. L., & Somero, G. N. (2011). Transcriptomic responses to salinity stress in invasive and native blue mussels (genus Mytilus). *Molecular Ecology*, *20*(3), 517–529. doi:10.1111/j.1365-294X.2010.04973.x

Louis, Y. D., Bhagooli, R., Kenkel, C. D., Baker, A. C., & Dyall, S. D. (2017). Comparative Biochemistry and Physiology , Part C Gene expression biomarkers of heat stress in scleractinian corals : Promises and limitations. *Comparative Biochemistry and Physiology, Part C*, *191*, 63–77. doi:10.1016/j.cbpc.2016.08.007

Lowe, M., Sehlinger, T., Soniat, T., & La Peyre, M. (2017). Interactive Effects of Water Temperature and Salinity on Growth and Mortality of Eastern Oysters, Crassostrea virginica: A Meta-Analysis Using 40 Years of Monitoring Data. *Journal of Shellfish Research*, *36*(3), 683–697.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*(1), 10. doi:10.14806/ej.17.1.200

Maynard, A., Bible, J. M., Pespeni, M. H., Sanford, E., & Evans, T. G. (2018). Transcriptomic responses to extreme low salinity among locally adapted populations of Olympia oyster (*Ostrea lurida*). *Molecular Ecology*, (510), 0–3. doi:10.1111/mec.14863

Meng, J., Zhu, Q., Zhang, L., Li, C., Li, L., She, Z., … Zhang, G. (2013). Genome and Transcriptome Analyses Provide Insight into the Euryhaline Adaptation Mechanism of Crassostrea gigas. *PLoS ONE*, *8*(3). doi:10.1371/journal.pone.0058563

Meng, X. L., Dong, Y. W., Dong, S. L., Yu, S. S., & Zhou, X. (2011). Mortality of the sea cucumber, Apostichopus japonicus Selenka, exposed to acute salinity decrease and related physiological responses: Osmoregulation and heat shock protein expression. *Aquaculture*, *316*(1–4), 88–92. doi:10.1016/j.aquaculture.2011.03.003

Meyer, E., Aglyamova, G. V., & Matz, M. V. (2011). Profiling gene expression responses of coral larvae (Acropora millepora) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. *Molecular Ecology*, *20*(17), 3599–3616. doi:10.1111/j.1365-294X.2011.05205.x

NOAA. (2007). Status Review of the Eastern Oyster (Crassostrea virginica) Report to the National Marine Fisheries Service , Northeast Regional Office, February 16, 2007. *Eastern Oyster Biological Review*, (March), 115.

NOAA. (2016). *NOAA Navigation Services Gulf of Mexico Activities Report*.

Ozsolak, F., & Milos, P. M. (2011). NIH Public Access. *Nature Reviews Genetics*, *12*(2), 87–98. doi:10.1038/nrg2934.RNA

Przeslawski, R., Byrne, M., & Mellin, C. (2015). A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. *Global Change Biology*, *21*(6), 2122–2140. doi:10.1111/gcb.12833

Przeslawski, R., Davis, A. R., & Benkendorff, K. (2005). Synergistic effects associated with climate change and the development of rocky shore molluscs. *Global Change Biology*, *11*(3), 515–522. doi:10.1111/j.1365-2486.2005.00918.x

R Development Core Team. (2013). A language and environment for statistical computing.

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. doi:10.1093/bioinformatics/btp616

Runcie, D. E., Garfield, D. A., Babbitt, C. C., Wygoda, J. A., Mukherjee, S., & Wray, G. A. (2012). Genetics of gene expression responses to temperature stress in a sea urchin gene network. *Molecular Ecology*, *21*(18), 4547–4562. doi:10.1111/j.1365-294X.2012.05717.x

Rybovich, M., Peyre, M. K. La, Hall, S. G., & Peyre, J. F. La. (2016). Increased Temperatures Combined with Lowered Salinities Differentially Impact Oyster Size Class Growth and Mortality. *Journal of Shellfish Research*, *35*(1), 101–113. doi:10.2983/035.035.0112

Sauvage, C., Boudry, P., De Koning, D. J., Haley, C. S., Heurtebise, S., & Lapègue, S. (2010). QTL for resistance to summer mortality and OsHV-1 load in the Pacific oyster (Crassostrea gigas). *Animal Genetics*, *41*(4), 390–399. doi:10.1111/j.1365-2052.2009.02018.x

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101–1108. doi:10.1038/nprot.2008.73

Schoville, S. D., Barreto, F. S., Moy, G. W., Wolff, A., & Burton, R. S. (2012). Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod Tigriopus californicus. *BMC Evolutionary Biology*, *12*(1), 170. doi:10.1186/1471-2148-12-170

Seneca, F. O., & Palumbi, S. R. (2015). The role of transcriptome resilience in resistance of corals to bleaching. *Molecular Ecology*, *24*(7), 1467–1484. doi:10.1111/mec.13125

Shimpi, G. G., Vargas, S., & Wörheide, G. (2016). Evaluation and validation of reference genes for qPCR analysis to study climate change-induced stresses in Sinularia cf. cruciata (Octocorallia: Alcyonidae). *Journal of Experimental Marine Biology and Ecology*, *483*, 42–52. doi:10.1016/j.jembe.2016.06.001

Shumway, S. E., & Koehn, R. K. (1982). Oxygen Consumption in the American Oyster Crassostrea virginica. *Marine Ecology - Progress Series*, *9*, 5–9.

Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology*, *53*(4), 597–608. doi:10.1093/icb/ict028

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G., & Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research*, *79*, 1–15. doi:10.1016/j.marenvres.2012.04.003

Somero, G. N. (2002). Thermal Physiology and Vertical Zonation of Intertidal Animals: Optima, Limits, and Costs of Living. *Integrative and Comparative Biology*, *42*(4), 780–789. doi:10.1093/icb/42.4.780

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." *Journal of Experimental Biology*, *213*(6), 912–920. doi:10.1242/jeb.037473

Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, *6*(11), 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x

Turner, R. E., Rabalais, N. N., & Justić, D. (2017). Trends in summer bottom-water temperatures on the northern Gulf of Mexico continental shelf from 1985 to 2015. *PLoS ONE*, *12*(9), 1–12. doi:10.1371/journal.pone.0184350

Wares, J. P., & Schiebelhut, L. M. (2016). What doesn't kill them makes them stronger: an association between elongation factor 1- α overdominance in the sea star *Pisaster ochraceus* and "sea star wasting disease." *PeerJ*, *4*, e1876. doi:10.7717/peerj.1876

Wright, R. M., Aglyamova, G. V., Meyer, E., & Matz, M. V. (2015). Gene expression associated with white syndromes in a reef building coral, Acropora hyacinthus. *BMC Genomics*, *16*(1), 1–12. doi:10.1186/s12864-015-1540-2

Zaugg, K., Yao, Y., Reilly, P. T., Kannan, K., Kiarash, R., Mason, J. Huang, P., Sawyer, S. K.,

Fuerth, B., Faubert, B., Elia, A., Luo, X., Nadeem, V., Bungard, D., Yalavarthi, S., Growney, J. D., Wakeham, A., Moolani, Y., Silvester, J., Ten, A. Y., Bakke, M. O. (2011). Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. *Genes & Development*, *25*(1), 1041–1051. doi:10.1101/gad.1987211.Freely

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., … Wang, J. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*, *490*(7418), 49–54. doi:10.1038/nature11413

Zhao, X., Yu, H., Kong, L., & Li, Q. (2012). Transcriptomic Responses to Salinity Stress in the Pacific Oyster Crassostrea gigas. *PLoS ONE*, *7*(9). doi:10.1371/journal.pone.0046244

Vita

Hollis Renee Jones, born in New Orleans, Louisiana, got her Bachelor of Science in 2015 in Biology at Northeastern University in Boston, MA. During her time at Northeastern she participated in the Three Seas Program studying marine biology at the Northeastern University Marine Science Center, Smithsonian Tropical Research Institute, and Friday Harbor Laboratories. She also worked as a Laboratory Assistant at the Marine Biological Laboratories in Woods Hole, MA, and as Giant Ocean Tank Assistant Aquarist at the New England Aquarium in Boston, MA. After her Bachelors she worked as a Marine Educator for the Pigeon Key Foundation and Marine Science Center in Pigeon Key, FL.

Hollis began her Masters of Science in 2016 at Louisiana State University where she became involved with Louisiana Sea Grant and in 2018 received the John A. Knauss Marine Policy Fellowship. The author plans to begin her fellowship position as a Research Integration Specialist in NOAA's Oceanic and Atmospheric Research (OAR) division in 2019.