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SYMPOSIUM

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

H. R. Jones, K. M. Johnson and M. W. Kelly[1]

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

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[1]E-mail: morgankelly@lsu.edu

Synopsis The eastern oyster, *Crassostrea virginica*, forms reefs that provide critical services to the surrounding ecosystem. These reefs are at risk from climate change, in part because altered rainfall patterns may amplify local fluctuations in salinity, impacting oyster recruitment, survival, and growth. As in other marine organisms, warming water temperatures might interact with these changes in salinity to synergistically influence oyster physiology. In this study, we used comparative transcriptomics, measurements of physiology, and a field assessment to investigate what phenotypic changes *C. virginica* uses to cope with combined temperature and salinity stress in the Gulf of Mexico. Oysters from a historically low salinity site (Sister Lake, LA) were exposed to fully crossed temperature (20°C and 30°C) and salinity (25, 15, and 7 PSU) treatments. Using comparative transcriptomics on oyster gill tissue, we identified a greater number of genes that were differentially expressed (DE) in response to low salinity at warmer temperatures. Functional enrichment analysis showed low overlap between genes DE in response to thermal stress compared with hypoosmotic stress and identified enrichment for gene ontologies associated with cell adhesion, transmembrane transport, and microtubule-based process. Experiments also showed that oysters changed their physiology at elevated temperatures and lowered salinity, with significantly increased respiration rates between 20°C and 30°C. However, despite the higher energetic demands, oysters did not increase their feeding rate. To investigate transcriptional differences between populations *in situ*, we collected gill tissue from three locations and two time points across the Louisiana Gulf coast and used quantitative PCR to measure the expression levels of seven target genes. We 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. In summary, oysters altered their phenotype more in response to low salinity at higher temperatures as evidenced by a higher number of DE genes during laboratory exposure, increased respiration (higher energetic demands), and *in situ* differential expression by season and location. These synergistic effects of hypoosmotic stress and increased temperature suggest that climate change will exacerbate the negative effects of low salinity exposure on eastern oysters.

Introduction

Average temperatures in the Gulf of Mexico (GOM) are rising in response to a changing global climate (Turner et al. 2017). Warmer temperatures result in more evaporation and regional changes in precipitation patterns (Biasutti et al. 2012). These changes have led to increased precipitation in the Southeastern USA and greater freshwater outflow through the two large rivers that flow into the GOM through the state of Louisiana. These rivers, the Mississippi and Atchafalaya, account for over 80% of the total freshwater input to the GOM, resulting in coastal Louisiana having the lowest near-shore salinities of the Gulf states (NOAA 2016). This combination of warming waters and increasing freshwater input is of special concern for marine invertebrates because simultaneous stressors may have greater effects than either stressor experienced individually (Przeslawski et al. 2005).

Multiple simultaneous stressors can constrain both plastic and evolutionary responses to global climate change (Somero 2010; DeBiasse and Kelly 2016;

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Kelly et al. 2017). Most marine invertebrates are osmoconformers that maintain internal osmolarities close to that of the surrounding seawater; this adaptation can increase sensitivity to prolonged immersion in low salinity conditions (Zhao et al. 2012). Temperature is also well known for influencing both the physiology and biogeographic distribution of sessile and benthic marine invertebrates (Guzman and Conaco 2016; Lancaster et al. 2016). The combined effects of temperature and salinity on marine invertebrates result in constraints derived from elevated energetic demands during stress exposure that manifest as increased physiological activity, costs of cellular maintenance, and damage repair (Somero 2002; Li et al. 2017). These cellular functions can compete with other critical biological functions such as reproduction and growth. Competing demands can lower the organism's ability to buffer against additional stressors such as fluctuations in food availability and parasitic infection.

Physiological trade-offs occur when two traits or functions cannot be simultaneously optimized, and may constrain the magnitude of an organism's plastic response to stress (Sokolova et al. 2012; Kelly et al. 2016). Synergistic effects of temperature and salinity—indicative of a physiological trade-off—have been documented for both gene expression and physiology in marine invertebrates (Przeslawski et al. 2005; Li et al. 2017). Heat stress can induce a shift from aerobic to anaerobic metabolic processes, leading to a buildup of toxic end products (Sokolova et al. 2012) or a downregulation of genes involved in energy metabolism in order to alleviate potential oxidative damage (Li et al. 2017). In marine invertebrates, the effects of heat stress can be exacerbated by extreme environmental salinities because low, or high, salinity conditions require the metabolically costly active transport of inorganic ions or organic osmolytes (Sokolova et al. 2012; Eierman and Hare 2014).

This study focused on the combined effects of heat and salinity stress on the eastern oyster, *C. virginica*, an ecologically, economically, and culturally important marine invertebrate inhabiting estuaries along the GOM (NOAA 2007). Over 80% of oyster reefs have been lost globally due to overfishing, disease, and loss of suitable habitat, caused in-part by lower environmental salinity conditions driven by increased precipitation and river outflows (Grabowski et al. 2012). Increased temperature and low salinities negatively impact both growth and survival in eastern oysters across all life stages (Rybovich et al. 2016; Lowe et al. 2017) and salinities below 5 PSU almost completely inhibit growth (LDWF 2016). Lowe et al. (2017) used 40 years of monitoring data to estimate that the optimal salinity for growth for all size classes of Louisianan oysters (spat, seed, and sack) is between 10.7 and 16.1 PSU. La Peyre et al. (2013) observed that *C. virginica* was able to survive low salinity exposure (below 3 PSU) when temperatures were under 25°C but experienced negative impacts on recruitment, survival, and growth in extended periods of low salinity during the hot summer months.

Because oysters are unable to properly maintain homeostasis in low salinity conditions under temperature stress, they rely on temporary physical isolation achieved through closing their valves. This response reduces the 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, and starvation (La Peyre et al. 2013). Therefore, the risk of mortality at high temperatures depends heavily whether it happens during a period of low salinity and how long the low salinity persists.

Oysters in the northern GOM experience the highest temperatures and the lowest salinities during the same time of year, June–August (Fig. 1). The effects of temperature and salinity on *C. virginica*'s growth and mortality (La Peyre et al. 2013; Rybovich et al. 2016; Lowe et al. 2017), physiology (Heilmayer et al. 2008; Casas et al. 2018b), and gene expression (Chapman et al. 2011) have individually been documented, but there is a growing need to link these phenotypic changes and physiological responses in order to fully understand the interacting effects of multiple stressors. In this study, we used a combination of comparative transcriptomics, physiological measurements, and field assessments to quantify the synergistic effects of combined temperature and salinity stress on adult *C. virginica*. First, we sequenced the transcriptomes of oysters from Sister Lake, LA that were exposed to fully crossed temperature (20°C and 30°C) and salinity (25, 15, and 7 PSU) treatments in the laboratory. Transcriptome-wide gene expression analysis can help quantify differences between individuals and populations that may not be reflected in other observable metrics of their physiology (Evans and Hofmann 2012). Second, we took physiological measurements of respiration and clearance rates for oysters at both temperatures (20°C and 30°C) at the lowest salinity (7 PSU) to mimic summer conditions. Respiration rates, or oxygen consumption, and clearance rate, the volume of water cleared of suspended particles per unit of time, can be used to approximate energetic demand and intake. Third, we conducted a

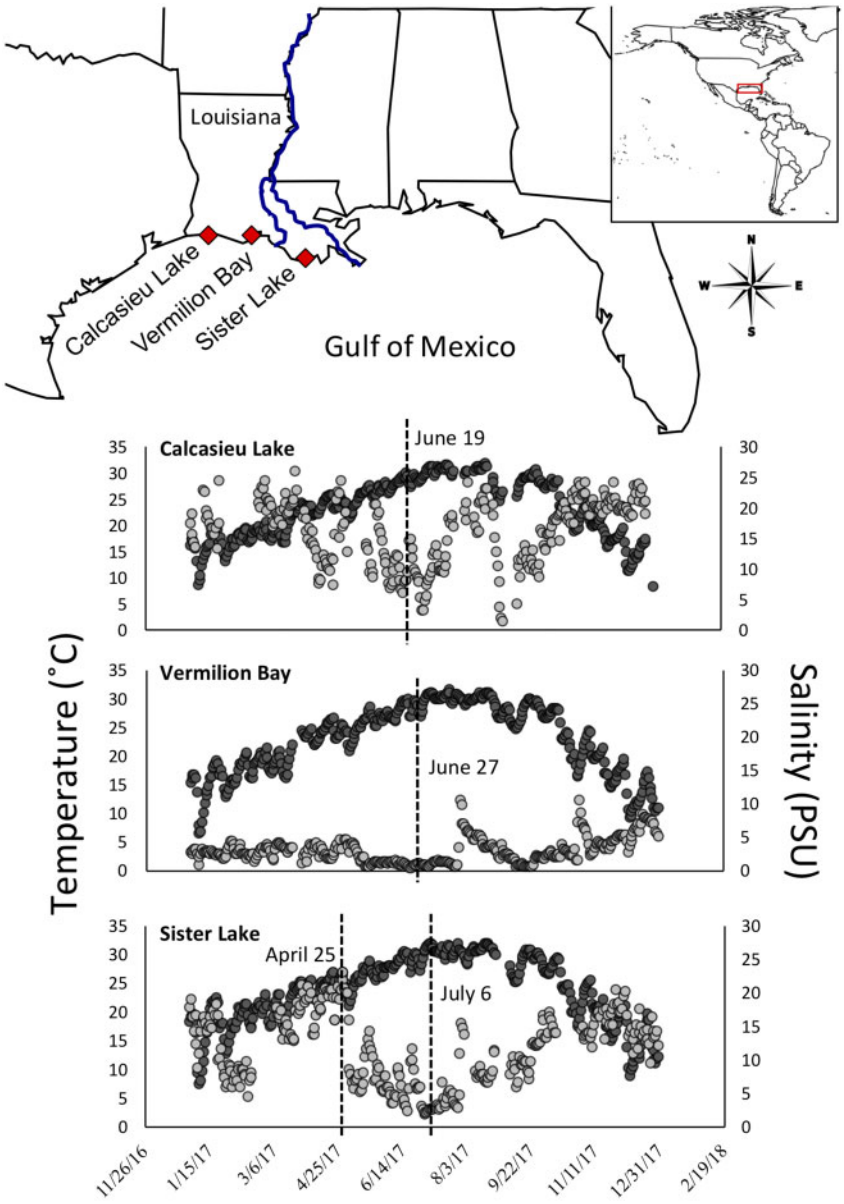

Fig. 1 Map of study area indicating collection sites: Calcasieu Lake, Vermilion Bay, and Sister Lake. The Mississippi and Atchafalaya Rivers are shown, with the Atchafalaya River as the western bifurcation of the Mississippi River. Scatter plots are mean daily salinities (PSU; light gray dots) and temperatures (°C; dark grey dots) for our three collection sites using data from USGS continuous data logger located as close to the oyster beds as possible. Perforated vertical lines represent collection dates. 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″.

field assessment to identify differential expression between seven genes that our analysis identified as potential target genes that respond to combined temperature and salinity stress. These *in situ* assessments of gene expression focused on comparisons across three sites with distinct annual salinity regimes along the Louisiana Gulf coast: Calcasieu Lake (mean annual salinity: 19.8 ± 5.6 PSU), Vermilion Bay (4.36 ± 3.21 PSU), and Sister Lake (12.2 ± 5.4 PSU) (see Supplementary Table S1 for more information on site temperatures and salinities).

We were especially interested in how physiological data line up with transcriptomic data and whether observed differences in the molecular response in the laboratory translate to *in situ* oyster gene expression. Our results reveal striking differences in the *C. virginica* response to low salinity at warmer temperatures in the GOM and emphasize that studies considering the impacts of multiple stressors, and

using multiple metrics of organismal performance are crucial for predicting the effects of climate change.

Materials and methods

Comparative transcriptomics

Oyster collection and laboratory exposure

We collected oysters for transcriptomic sequencing at two time points. In August 2015, ~350 oysters were dredged from Sister Lake, LA (29°14′57″N, 90°55′16″W) and maintained at Louisiana Sea Grant's Oyster Research Lab in Grand Isle, LA. All oysters were estimated to be around 1.5 years old and 70.3 ± 7.2 mm in shell height and were part of a larger study (Casas et al. 2018a). After 3 months of acclimation at the Oyster Research Lab, these oysters were transported to the Louisiana State University (LSU) in Baton Rouge, LA for exposure. The second collection was completed in April 2017, where ~100 oysters were dredged from Sister Lake, LA and directly transported to LSU for acclimation and exposure. Oysters from this collection had mean shell heights of 97.7 ± 13.2 mm.

For both collections oysters were pressure washed, scrubbed, and all biofoulers (barnacles and mussels) were removed. To reach experimental conditions, the treatment salinities and temperatures were adjusted at a rate of three units per day, every other day until they arrived at their experimental values. They were maintained at these experimental values for 4 weeks before sampling in order to mimic long-term summer exposure. The 2015 oysters were exposed to fully crossed temperature treatments of 20°C (cool) and 30°C (warm) and salinity treatments of 15 PSU (medium) and 25 PSU (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 7 PSU (low) (Supplementary Table S1.2). Oysters were kept in 700-L tanks with recirculating artificial seawater. Details on oyster laboratory maintenance in Supplementary Methods S1.1.

We choose these treatments based on information from US Geological Survey (USGS) continuous data recorders near the collection sites. Sister Lake, LA has a 2006–2016 year-round average temperature of 22.8 ± 5.4°C and salinity of 12.2 ± 5.4 PSU (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 (Lowe et al. 2017). As we did not want these oysters to close their valves and cease feeding during the experiment, we chose 7 PSU salinity as our low salinity treatment. We chose 15 PSU salinity because it falls within optimal condition windows and has not been shown to negatively affect physiological functioning, and while 25 PSU is also considered to be an optimal salinity for oyster growth it mimics higher salinity estuaries found in the same region (LDWF 2016).

After 4 weeks of acclimation to these treatments, four oysters were haphazardly selected from each treatment (16 oysters from the 2015 exposure and 8 oysters from the 2017 exposure: $n = 24$) were sacrificed and 4 mm × 4 mm gill tissue samples were excised and stored in RNA*later* (Thermo Fisher Scientific, Waltham, MA) for RNA sequencing.

RNA-seq library preparation, sequencing, and quality filtering

Individual-specific libraries for each oyster were multiplexed and sequenced across three lanes of an Illumina HiSeq3000 using 100 bp paired-end sequencing for the 16 2015 oysters, and 100 bp single-end sequencing for the 8 2017 oysters (Illumina, Inc., San Diego, CA) at Iowa State University's DNA Sequencing Facility (Ames, IA). Reads were quality checked using FastQC (v.0.11.5) (Andrews 2010), trimmed as needed for adapters using Trimmomatic (v.0.38) (Bolger et al. 2014), and reads shorter than 36 bp were discarded. Additional library preparation details can be found in Supplementary Methods S1.2.

Transcriptome assembly, read mapping, and differential expression analysis

Quality controlled forward reads from the 2015 paired-end data and the 2017 single-end reads were mapped to the published transcriptome for *C. virginica* (NCBI accession: GCF_002022765.2 C_virginica-3.0) using RSEM (v.1.3.0) (Li and Dewey 2011). Differentially expressed (DE) genes were identified using the edgeR (v.3.20.7) package (Robinson et al. 2010). All analyses were completed using R (v.3.4.2) (R Core Team 2017). 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 generalized linear model (GLM) for subsequent pairwise comparisons between treatment groups. Pairwise comparisons were done using the glmLRT function, a likelihood ratio test, that considers a GLM of the negative binomial distribution family to test for significant differential expression. The individual oyster libraries were identified by their treatment temperature (30°C as W and 20°C as C) and treatment salinity (25 PSU as H, 15 PSU as M, 7 PSU as L)

(Supplementary Table S1.2). 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. The R code used for the differential expression analysis can be found at https://github.com/hrjones10/jones_edgeR.

We used the R package vegan (v.2.5.2) (Anderson 2001) to perform a principal coordinate analysis (PCoA) by calculating Euclidean distances to visualize clustering of gene expression between the temperature and salinity treatments; significance was calculated using a PERMANOVA with the adonis function. Batch effect was removed for all analyses.

Annotation and functional enrichment analysis

As the reference transcriptome for *C. virginica* is still in development, we added gene ontology (GO) terms to the reference using the sequence annotations available with the latest release of the *C. virginica* genome (GCF_002022765.2, version 3.0). These transcriptome annotations were combined with GO terms generated using InterProscan 5 (v 5.27-66.0) and the most recent GO database release (format-version: 1.2, release: 2018-02-20).

After annotation, we were able to compare individual responses to identify overlap in genes that were upregulated or downregulated between treatments. We performed a Mann–Whitney *U* test in R using the package ape (v.5.2) to identify up or downregulated functionally enriched biological process (BP) GO terms in our pairwise comparisons (Wright et al. 2015). Further information, scripts, and examples files are available at https://github.com/z0on/GO_MWU.

Physiology

For the oysters collected in Spring 2017 and exposed to the treatment temperatures 20°C and 30°C at the low salinity treatment (7 PSU) that were not sacrificed for RNA-seq analysis, we measured both clearance rate (L h^{-1}) ($n = 40$) and respiration rate (mg O_2 h^{-1}) ($n = 50$). Because the 2015 oysters had all been sacrificed before the conception of the 7 PSU addition, we are only able to report on physiological measurements from the 2017 exposure.

Respiration rates were measured for open oysters in a respirometer until the oxygen percentage fell below 70% saturation (typically less than 90 min), and standardized using dry meat weight (g), which was measured after drying at 70°C for 48 h. Clearance rate for open oysters was measured over a period of 40 min and standardized using shell heights (mm). Oysters used for clearance rate and respiration rate measurements were starved for 1-week prior to measurement to ensure that we measured their standard or basal metabolic rates (Shumway and Koehn 1982). Because RNA-seq tissue sampling is lethal and this starvation period can affect gene expression, we did not use the same oysters for both RNA-seq and the physiological measurements. Additional methods in Supplementary Methods S1.3 and S1.4.

Genes of interest

Oyster collection

In the summer of 2017 we excised and stored Louisianan *C. virginica* gill tissue in RNA*later* from adult oysters in order to measure targeted *in situ* gene expression. Individuals were manually dredged from Sister Lake on two different time points (A: 4/25/17, $n = 15$; B: 7/6/17, $n = 15$), Vermilion Bay (6/27/17, $n = 15$), and Calcasieu Lake (6/19/17, $n = 16$). Gill tissue was excised and stored within minutes of dredging in order to minimize changes in gene expression associated with handling. The tissue from these oysters was used in quantitative PCR (qPCR) analyses to test for variation in gene expression among sites and sampling times that differed in salinity and temperature. Temperature and salinity measurements were simultaneously taken at each site using an YSI (Fig. 5).

Target genes expression analysis

Expression of seven target genes (carnosine synthase, elongation factor 1-alpha, heat shock protein 70, anti-apoptotic protein, cadherin, palmitoyltransferase, and sodium hydrogen exchanger) and one housekeeping gene (myosin light chain kinase) was measured using qPCR. Additional information on target gene selection, primer design, and primer sequences can be found in Supplementary Methods S1.5 and S1.6 and Supplementary Table S1.4.

To test for expression differences, we generated cDNA from the 62 preserved gill tissue samples and measured each gene's expression level in triplicate across two 96-well qPCR plates (31 individuals per plate). Expression levels were normalized against the expression of the housekeeping gene myosin light chain kinase and expression differences across individuals and populations were calculated using the delta delta Ct ($2^{-\Delta\Delta ct}$) method and *z*-scores calculated to normalize expression variation across genes (Schmittgen and Livak 2008). We used the R package pheatmap (v.1.0.10) to visualize expression data. A Kruskal–Wallis test was performed for each gene across the three populations and two time points to test for significant differences in expression patterns.

We used the R package vegan (v.2.5.2) (Anderson 2001) to perform a PCoA to visualize clustering of expression patterns for the four genes that were most different among samples: sodium hydrogen exchanger, carnosine synthase, anti-apoptotic protein, and elongation factor 1-alpha by location; significance was calculated using a PERMANOVA with the adonis function.

Results

Comparative transcriptomics

Transcriptome assembly and read mapping

Sequencing of the pooled libraries ($n = 24$) resulted in an average of 34.1 ± 20.7 million paired-end reads per library in the two lanes with 12 individuals per lane (one of the lanes was split with another study) and an average of 43.5 ± 10.9 million single-end reads in the lane with 8 individuals. After mapping to the publicly available *C. virginica* transcriptome we found one library with a low mapping rate of 25.9%. After a BLAST query of the top overrepresented sequenced we determined that there were high levels of rRNA contamination in the sample and discarded it from further analysis (treatment group: WH). The other 23 transcriptomes aligned to the published transcriptome with a weighted average of $71.89 \pm 1.6\%$. Of this total alignment, an average of $39.2 \pm 3.15\%$ of the transcripts aligned to multiple places in the transcriptome (the rest being unique alignments: average $31.8 \pm 2.3\%$).

Differential expression

The nine pairwise comparisons performed using the GLM of the negative binomial family in edgeR (v3.20.7) identified a total of 2356 transcripts as significantly DE with a FDR ≤ 0.05. A PCoA (Fig. 2) of all DE genes (including insignificantly DE) revealed significant clustering by both temperature (adonis $P < 0.05$) and salinity (adonis $P < 0.005$). Standard deviation ellipses were more tightly clustered for the low salinity and the cooler temperature, indicating less variation among individuals for these treatments. Finally, we found that the majority of transcripts had greater fold change in expression at the warmer temperature than the cooler temperature (Fig. 4; 732 out of 1027 transcripts, Fisher's exact test, $P < 0.0001$). All pairwise DEG results are available in Supplementary Table S2.

Functional enrichment

Functional enrichment analyses of the DE transcripts from each pairwise comparison allowed us to identify enriched GOs associated with the GO category, Biological Process. In Table 1, we list the top 10 DE GO terms that contained <1000 sequences for the three comparisons that represent changes due to hypoosmotic and thermal stress: WM vs. WL, CM vs. WM, and CM vs. CL. Eight GO terms overlapped between the WM vs. WL and CM vs. WM comparisons so 22 terms are represented. For example, the GO term "cell–cell adhesion" is strongly upregulated in response to low salinity at the warmer temperature but strongly downregulated in response to temperature stress at the control salinity (Table 1). The CM vs. CL comparison only had one significant GO term, G-protein coupled receptor signaling pathway, which was downregulated in response to low salinity. All GO enrichment results are available in Supplementary Table S3.

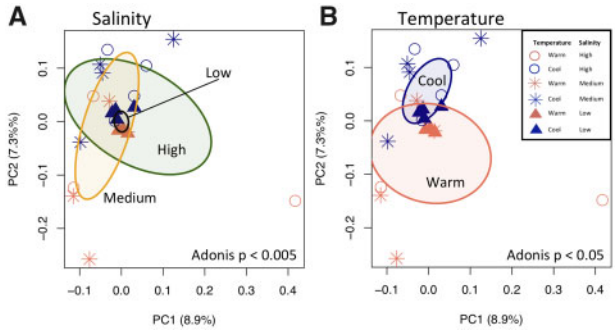

Fig. 2 PCoA of the 23 individual *Crassostrea virginica* samples' differential gene expression. Ellipses are standard deviations around each treatment. **A**) Clustering of the three salinity levels (adonis $P < 0.005$). **B**) Clustering of the two temperature levels (adonis $P < 0.05$).

Physiology

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 $P = 5.76e{-}12$, $F_{1,48} = 82.11$) (Fig. 4). There was no significant effect of temperature on 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 $P = 0.356$, $F_{1,38} = 0.871$) (Fig. 4). Individual clearance rates, respiration rates, shell heights, and dry meat weights are shown in Supplementary Table S1.5.

Field assessment

A heatmap of the z-scored $2^{-\Delta\Delta ct}$ qPCR results for the seven target genes was used to visually inspect expression profiles (Fig. 5). Particularly evident is the upregulation of EF1A, an anti-apoptotic protein,

Table 1 Top 10 significantly DE GO terms for the three treatments WM vs. WL, CM vs. CL, and CM vs. CL and their regulation (+ for upregulated, − for downregulated), number of sequences expressed as a fraction, and adjusted *P*-value for each comparison

		WM vs. WL			CM vs. WM			CM vs. CL			
Top term in:	**GO term**	**Regulation**	**Nseqs**	***P* adj**	**Regulation**	**Nseqs**	***P*-adj**	**Regulation**	**Nseqs**	***P* adj**	**GO ID**
WM vs. WL and CM vs. WM	Cell–cell adhesion	−	43/78	****	+	44/78	****				GO: 0007156; GO: 0098742; GO: 0098609
	Cell adhesion	−	64/123	***	+	65/123	**	+			GO: 0007155; GO: 0022610
	Protein–DNA complex assembly	+	20/28	***	−	21/28	****				GO: 0006334; GO: 0034728; GO: 0065004; GO: 0071824
	RNA biosynthetic process	+	22/41	***	−	24/41	***	+			GO: 0032774
	RNA metabolic process	+	52/136	**	−	52/136	***	+			GO: 0016070
	Transmembrane transport	−	188/463	**	+	177/463	***	+			GO: 0055085
	Cellular protein-containing complex assembly	+	27/44	**	−	27/44	****	+			GO: 0034622
	Chromatin organization	+	31/50	*	−	34/50	****	+			GO: 0006325
WM vs. WL	Organic cyclic compound biosynthetic process	+	38/81	**	−	40/81	**	+			GO: 0034654; GO: 0018130; GO: 0019438; GO: 1901362
	Ion transport	−	137/324	**				+			GO: 0006811
CM vs. WM	Macromolecule biosynthetic process	+	66/137	*	−	66/137	***	−			GO: 0009059
	Cellular macromolecule biosynthetic process	+			−	38/98	**	−			GO: 0034645
CM vs. CL	G-protein coupled receptor signaling pathway	+	216/582	*	−			+	175/582	****	GO: 0007186
	Transposition	−			−			−			GO: 0006313; GO: 0032196
	Movement of cell or subcellular component	−			−			−			GO: 0006928; GO: 0007018
	Oxoacid metabolic process	−						−			GO: 0019752; GO: 0043436; GO: 0006082
	Protein ubiquitination	+						+			GO: 0016567; GO: 0032446

Positive regulation of response to stimulus	+			−			−	GO: 0042742; GO: 0009617; GO: 0050829; GO: 0048584; GO: 0050708; GO: 0002791; GO: 0051223; GO: 1903530; GO: 0051046; GO: 0090087; GO: 0070201; GO: 0032880; GO: 0042116; GO: 0002274; GO: 0045321; GO: 0001775; GO: 0002755; GO: 0002224; GO: 0002221; GO: 0002758; GO: 0002218; GO: 0002757; GO: 0002253; GO: 0045089; GO: 0002764; GO: 0050778; GO: 0050776; GO: 0002684; GO: 0031349; GO: 0045088; GO: 0031663; GO: 0050707; GO: 0034142
Positive regulation of cell cycle	+			+	4/9	*	+	GO: 0045787; GO: 0071157; GO: 0071156
Microtubule-based process	−	31/67	*	+			−	GO: 0007017
Tricarboxylic acid metabolic process							−	GO: 0006099; GO: 0006101; GO: 0072350
Negative regulation of cell death	+			+			+	GO: 0060548; GO: 0043066; GO: 0043069

If the term was not significantly expressed in one of the comparisons it is left blank. The regulation refers to the second term in the comparison (e.g., cell–cell adhesion is downregulated in the WL compared with the WM).
*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.00005$.

carnosine synthase, and a sodium hydrogen exchanger at the lower salinity location (Vermilion Bay), and the low salinity sampling time-point at Sister Lake (B). For the two Sister Lake sampling time points, B is considered to be the low salinity time point because the salinity was 2.3 PSU when we collected oysters, compared with A which had a salinity of 19.1 PSU. Results from the Kruskal–Wallis tests indicated that each gene was DE in at least one of the four locations or time points (HSP70 $P = 0.02$, palmitoyltransferase $P = 0.02$, 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 $P = 4.3e{-}08$). The expression levels of the seven target genes in the transcriptome assembled from our laboratory exposed oysters and primer information in Supplementary Table S1.4.

Discussion

Our study examined the effects of long-term combined temperature and salinity stress—two important and regularly fluctuating stressors in the coastal marine environment—on the transcriptomic and physiological responses of the eastern oyster, *C. virginica*. Because of the necessarily dynamic nature of transcriptomics, we acknowledge that comparisons made between individuals exposed in different years and collected at different times need to be taken with a grain of salt, despite the application of identical methods. To increase the strength of our analysis, we used multiple approaches and found that temperature and salinity have non-additive effects on *C. virginica*, with the effects of the two stressors being greater than the sum of their individual effects (Przeslawski et al. 2015). When *C. virginica* was exposed to low salinities (7 PSU) at warmer temperatures (30°C) the magnitude and number of DE genes increased (Fig. 3). Oysters exposed to low salinities also presented low levels of variation in gene expression among individuals, regardless of temperature, compared with individuals from medium and high salinities (Fig. 2). Functional enrichment analysis revealed that significantly DE genes were associated with GO terms that frequently had opposite regulation for hypoosmotic and thermal stress (Table 1). Additionally, we saw low overlap in the enriched GO terms between low salinity at the warm and cool temperatures (Table 1). This indicates that the responses to each individual stressor rely on distinct response pathways. Modulation of energy metabolism is key in the cellular stress response (Kültz 2005) and the energetic demands required to maintain pathways involved in the temperature and salinity stress response can result in energetic trade-offs.

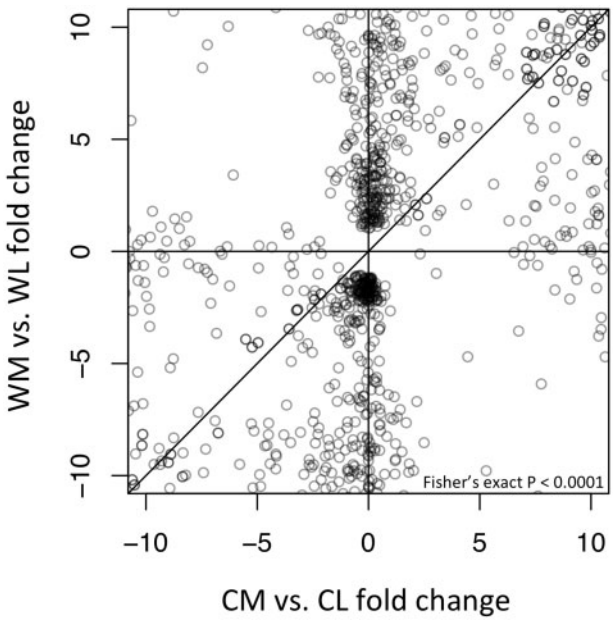

Fig. 3 Log fold change in expression for significantly DE transcripts in WM vs. WL and CM vs. CL pairwise comparisons. The one-to-one line divides upregulated transcripts with greater fold change in expression at the warmer temperature compared with the cooler temperature. Transcripts that were more strongly downregulated at the warmer temperature fall below the one-to-one line but to the left of the y-axis. Transcripts that were more strongly upregulated at the warmer temperature fall above the one-to-one line but to the right of the y-axis. vertical line. When we add the transcripts that fall into these quadrants we find 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 $P < 0.0001$). WM vs. WL refers to the 30°C, 15 PSU vs. 30°C, 25 PSU comparison and CM vs. CL refers to the 20°C, 15 PSU vs. 20°C, 15 PSU comparison.

Physiological measurements taken only at the low salinity demonstrated that respiration rate increased in the warmer temperature but clearance rate did not (Fig. 4), suggesting an energetic gap. Our results fall within the range of published rates for GOM *C. virginica* (Casas et al. 2018b). Respiration rate can be used to estimate metabolic costs, and clearance rates can be used to estimate the amount of energy ingested over time (Casas et al. 2018b); both are important when considering energetic trade-offs imposed by combined temperature and salinity stress. A reduction of energy intake coupled with increased energetic demands during the hypo-osmotic stress response could lead to increased mortality in oysters exposed to these combined stressors (La Peyre et al. 2013).

We saw significant differences in expression of our seven target genes in our qPCR analysis across

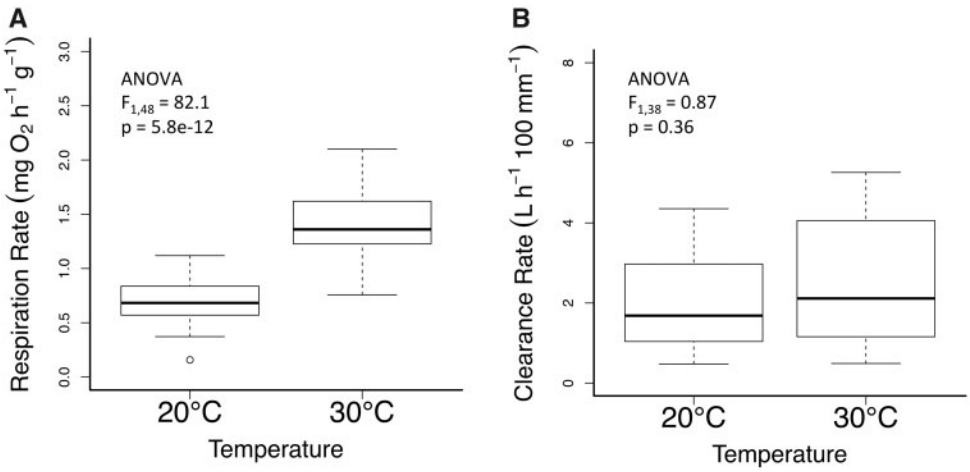

Fig. 4 A) Respiration rate (mg O_2 h^{-1}) and **B**) clearance rate (L h^{-1} 100 mm^{-1}) for the two temperature treatments 20°C and 30°C at 7 PSU. There is a significant effect of temperature on respiration rate (ANOVA $P = 5.76e{-}12$, $F_{1,48} = 82.11$) but no significant effect of temperature on clearance rate (ANOVA $P = 0.356$, $F_{1,38} = 0.871$).

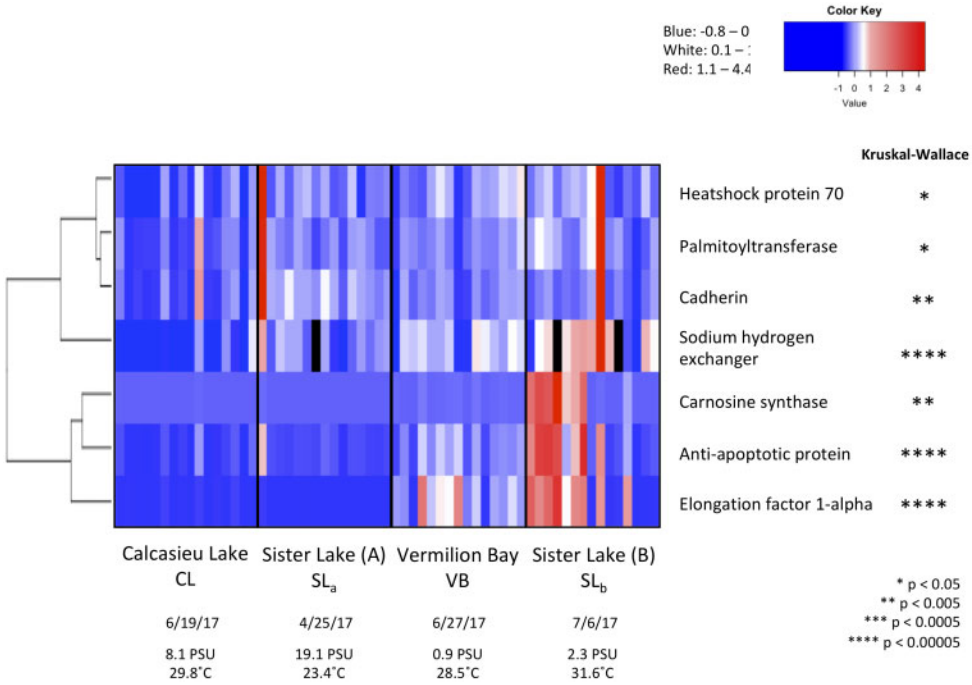

Fig. 5 Heat map of *z*-scored $2^{-\Delta\Delta ct}$ values for four target genes. Three locations and two time points shown: Calcasieu Lake (CL), Vermilion Bay (VB), Sister Lake A (SL_a), and Sister Lake B (SL_b) with the mean salinity and temperature on the day they were collected. The low salinity location is Vermilion Bay and the low salinity collection day is Sister Lake B. Black bars are N/As. *P*-values from a four-way Kruskal–Wallis show significant differences between the four sampling events for each gene.

Calcasieu Lake, Vermilion Bay, and the two time points at Sister Lake (A and B). Surprisingly, we found that EF1A, a protein synthesis gene that is typically considered a housekeeping gene (Morga et al. 2010) showed significant upregulation in the two lower salinity oyster collection: Vermilion Bay and Sister Lake B (Fig. 5). Housekeeping genes are generally constitutively expressed genes involved in basic cellular maintenance and do not change expression levels in different environmental conditions. As such, we propose that EF1A is not reliable for this purpose in *C. virginica*. Both the Vermilion Bay and Sister Lake B sampling events took place at times when salinities were below 7 PSU, potentially explaining why we saw changes in the expression of EF1A in field samples but not in the transcriptomic data. Additionally, Vermilion Bay had been experiencing salinities below 7 PSU for over a month

before sampling, while Sister Lake B had only dropped below 7 PSU the week of sampling. We were also surprised by the lack of signal observed in HSP70 expression but predict that it could be due to the pattern observed in Meyer et al. (2011) where similar heat shock proteins were upregulated in the short-term temperature stress treatments and down-regulated in long-term exposures.

Synergistic effects of temperature and salinity stress

Oysters in the northern GOM experience the lowest salinities in the summer because of increased rainfall and outflow from the Mississippi and Atchafalaya Rivers. While low salinity events in estuaries typically occur as brief pulses, future changes in salinity regimes may lead to longer duration low salinity events, as in the case of opening of diversions, or larger storms, which in in this region are capable of producing extended low salinity events. When oysters are faced with changes in salinity they need to i) regulate inorganic and organic osmolytes to maintain cell volume (at a metabolic cost) or, at extremely low salinities (below 5–3 PSU), ii) isolate themselves from the water column by closing their valves. Our RNA-seq analysis revealed that the GO term "transmembrane transport" was significantly enriched among the upregulated transcripts in response to low salinity at the warmer temperature (WM vs. WL). Surprisingly, this term was also enriched among the downregulated transcripts in response to the warmer temperature at the medium salinity (WM vs. WL) (Table 1). Similar results have been described by Lockwood and Somero (2011), who measured the transcriptomic response to salinity stress in two *Mytilus* species, also marine bivalves, and compared their results with a 2010 study that characterized the transcriptomic response of these same two mussel species to heat stress (Lockwood et al. 2010). They found that of the 45 genes DE in response to both stressors, the genes most strongly upregulated by heat stress were most strongly downregulated by hypoosmotic stress. The GO term "ion transmembrane transporter activity" was also significantly over-represented in those 45 genes, possibly because upregulation of ion transporters allows ions to move more readily across cell membranes to equalize concentrations on either side; however, downregulation stops the transport of solutes into the cytosol, where the solute concentrations are already too high. In addition, the increased permeability of lipid membranes during heat stress (Kültz 2005) may decrease the efficacy of active ion transport (Kelly et al. 2016). These changes may explain why we saw opposite regulation of all of the significantly DE GO terms represented in Table 1 between the WM vs. WL and CM vs. WM comparisons.

The GO term "G-protein coupled receptor signaling pathway" was significantly enriched among the downregulated transcripts in response to low salinity at both 20°C and 30°C (Table 1 shows the WM vs. WL and CM vs. CL comparisons). This GO term is a subset of the parent category "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 and Hare 2014). The downregulation of these genes could indicate that oysters are trying to match the solute concentrations of their surroundings at low salinities. 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 (Meng et al. 2013; Eierman and Hare 2014).

The GO term "microtubule-based process" was significantly enriched among upregulated transcripts in the low salinity treatment for both 20°C and 30°C (Table 1) with no significant change for the medium salinity comparison. 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 et al. 2018).

Whether an oyster's valve is open or closed, maintaining internal homeostasis in the face of changing environmental conditions is energetically costly. We did not record the duration or frequency of valve closure during treatment exposure but, because the treatments were designed not to kill the oysters, but rather place them in survivable conditions, we suggest that the valves were open for feeding and therefore exposing them to external environmental conditions.

Future directions

Future coastal management plans for the northern GOM include sediment and freshwater diversions, which would introduce freshwater from the Mississippi River into estuarine environments to ameliorate the effects of coastal erosion (CPRA 2017). The synergistic effects of low salinity and high temperature stress observed here suggest that it is important to consider the seasonality of these management activities, and that efforts should be made to prevent freshwater releases coinciding with

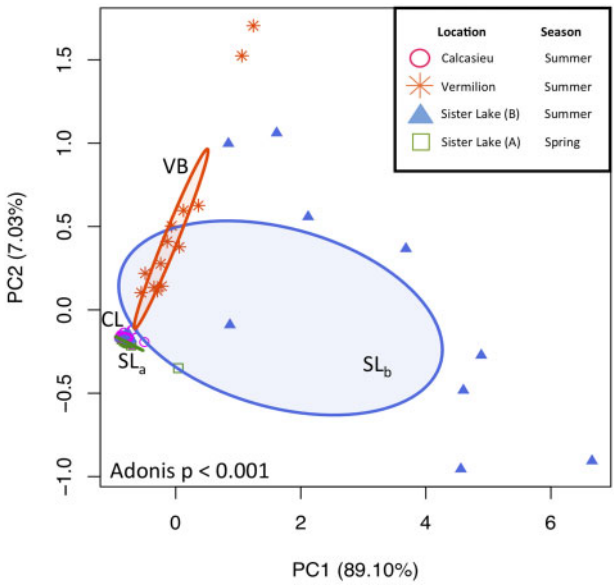

Fig. 6 PCoA of the $2^{-\Delta\Delta ct}$ for the four genes sodium hydrogen exchanger, carnosine synthase, anti-apoptotic protein, and elongation factor 1-alpha. Ellipses are standard deviations around each location (adonis $P < 0.001$).

temperatures >30°C, in order to improve survival of estuarine oyster beds (La Peyre et al. 2013).

There is a need for the ability to assess oyster condition in the field after a stressful event like a hurricane or freshwater diversion. The PCoA of expression for the four genes measured via qPCR indicates a break along PC1 where the low salinity sampling (Vermilion Bay and Sister Lake in the summer) separate from the high salinity sampling (Calcasieu Lake and Sister Lake in the spring) (Fig. 6). This suggests that expression of these four genes could be used for rapid identification of oysters that have recently experienced combined temperature and hypoosmotic stress in the field. The ability to quickly classify oyster condition using gene expression profiles would be useful for monitoring efforts but would benefit from additional data to confirm the patterns described here.

Conclusions

Phenotypic plasticity in response to environmental variability has important implications for a population's tolerance to biotic and abiotic stressors. As tolerances shift, we may begin to see populations pushed closer to tolerance thresholds for a variety of stressors. We tested for physiological trade-offs in *C. virginica* in response to temperature and salinity, using transcriptome-wide gene expression data, physiology experiments, and qPCR. Our results suggest that timing and duration of freshwater events will have large impacts on eastern oyster recruitment and survival as temperatures gradually warm and push them closer to their tolerance thresholds. Linking environment, physiology, and gene expression profiles provided a more complete picture of how climate change will impact these phenotypically plastic organisms. Temperature and salinity are only two of many shifting environmental variables and the cascading and sometimes unpredictable impacts of multiple stressors is why studies that measure responses to multiple variables will be key to predicting the effects of future environmental change.

Data accessibility

Raw sequences are available on the NCBI SRA database under BioProject ID PRJNA508205. The R code used for the differential expression analysis can be found at https://github.com/hrjones10/jones_edgeR.

Author contributions

H.R.J. collected and analyzed the data and led the writing of the manuscript. K.M.J. analyzed the data and contributed to the writing of the manuscript. M.W.K. conceived the idea, designed the methodology, and contributed to the writing of the manuscript.

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Supplementary data

Supplementary data are available at *ICB* online.

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