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Transcriptomic signatures of temperature adaptation in the eastern oyster *Crassostrea virginica*

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Abstract

The large geographic distribution of the eastern oyster, *Crassostrea virginica*, makes it an ideal species to test how populations have adapted to latitudinal gradients in temperature. Despite inhabiting distinct thermal regimes, populations of *C. virginica* near the species' southern and northern geographic range show no population differences in their physiological response to temperature. In this study, we used comparative transcriptomics to understand how oysters from either end of the species' range maintain enantiostasis across three acclimation temperatures (10, 20, and 30°C). With this approach, we identified genes that were differentially expressed in response to temperature between individuals of *C. virginica* collected from New Brunswick, Canada and Louisiana, USA. We observed a core set of genes whose expression responded to temperature in both populations, but also an even larger set of genes with expression patterns that were unique to each population. Intriguingly, the genes with population-specific responses to temperature had elevated F_{ST} and Ka/Ks ratios compared to the genome-wide average. In contrast, genes showing only a response to temperature were found to only have elevated F_{ST} values suggesting that divergent F_{ST} may be due to selection on linked regulatory regions rather than positive selection on protein coding regions. Taken together, our results suggest that, despite coarse-scale physiological similarities, natural selection has shaped divergent gene expression responses to temperature in geographically separated populations of this broadly eurythermal marine invertebrate.

KEYWORDS

adaptation, *Crassostrea virginica*, gene expression, mollusks, organism-environment interactions, oysters

1 | INTRODUCTION

When an organism is capable of inhabiting a broad range of environmental conditions, comparisons among populations from the extreme ends of its geographic range can provide insights into how such broad tolerance is possible. In some cases, broad tolerance is achieved with a single 'general purpose' phenotype, capable of surviving all conditions (Baker, 1965). In other cases, broad tolerance at the species level is achieved through local adaptation, where a series of populations are each optimized to their individual conditions, collectively comprising a species with broad tolerance (Bolnick et al., 2003; Kawecki & Ebert, 2004). Understanding the mechanisms underlying a broad thermal tolerance is critical to understanding how populations will respond to increasing sea surface temperatures across a species range.

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Even for a euryhaline invertebrate, the eastern oyster (*Crassostrea virginica*) inhabits an especially broad range of conditions. Its geographic range extends from the Gulf of St. Lawrence, Canada, to the Gulf of Mexico and Caribbean Sea, where it inhabits estuaries with salinities ranging from 4 to 35 PSU, and annual water temperatures ranging from −2°C to 36°C (Shumway, 1996). In the shallow estuaries of the Gulf of St. Lawrence, water temperatures range between −1.5°C and 22°C, where temperatures above 5°C are considered to be the minimum temperature for feeding by *C. virginica*, which occurs for only about half of the year (Comeau et al., 2012). This is in contrast to the already warm temperatures in the northern Gulf of Mexico (nGOM) that range between 9°C and 32°C, allowing for feeding year-round (Casas et al., 2015, 2017; Lowe et al., 2017). However, *C. virginica* populations in the nGOM regularly experience temperatures >30°C, which inhibit growth and drive increased mortality, especially at lower salinities, which often coincide with warmer temperatures in this region (Jones et al., 2019; Lowe et al., 2017; Rybovich et al., 2016). In addition, recent models suggest the Gulf of St. Lawrence is one of the world's fastest warming regions as a result of a weaker Atlantic meridional overturning circulation (Caesar et al., 2018; Claret et al., 2018). This presents a developing stressor for eastern oysters in the Gulf of St. Lawrence and reinforces the need to understand the degree of adaptation to temperature between these two regions. As such, we have sought out to test whether populations from Louisiana (LA) have adaptations to warmer temperatures whereas individuals from New Brunswick (NB) may harbour adaptations to cold environments.

To investigate the mechanisms underlying this species' broad temperature tolerance, we built on prior physiological experiments and examined the transcriptomic response to temperature in these populations from close to the extremes of the geographic range of this species: Bay Gardene and Sister Lake in Louisiana, USA, and Baie Saint-Simon-Sud, New Brunswick, CA. We used RNA-seq to measure the global gene expression after 4 weeks of acclimation to 10, 20, and 30°C in oysters from each of these two regions. For our analysis, we considered all individuals from Louisiana as one population (LA) and all individuals from New Brunswick as a second population (NB). The previously published physiological measurements indicate that oysters from these two regions alter their feeding and respiration rates at these temperatures in broadly similar ways, with differences between the LA and NB populations in oxygen consumption rates and condition index, but no population x temperature interactions for any physiological measurements (Casas et al., 2018). In general, Casas et al. (2018) found consistent evidence for decreased feeding rates at 10°C, a steady increase in oxygen consumption rates from 10°C to 30°C, and that the 20°C treatment was marked by oysters spending the greatest amount of time with valves open (indicative of low stress). These previously published results underscore that 20°C treatment is the least stressful of the three treatment conditions (Table 1).

Although there was no significant effect of population on these physiological measurements, previous research has suggested that populations can achieve the same canalized phenotype through different underlying mechanisms (Takahashi, 2019). Comparing gene expression responses at each temperature therefore allows a fine-scaled test of how physiological responses to temperature have diverged under long-term evolutionary histories in different thermal environments. A substantial number of studies have made this comparison for populations that differ in their history of exposure to thermal stress (Porcelli et al., 2015). Some species exhibit very similar responses to temperature among geographically separated populations (*Drosophila* (Cooper et al., 2012; Clemson et al., 2016), *Anolis* (Campbell-Staton et al., 2018)). However, the majority of transcriptomics studies have observed a gene expression response to temperature that is qualitatively similar but differs in magnitude between populations. In some cases, the response is greater in the more heat tolerant population: whitefly, *Bemisia tabaci* (Mahadav et al., 2009), trout, *Oncorhynchus mykiss* and *Salmo trutta* (Meier et al., 2014; Narum & Campbell, 2015), stickleback, *Gasterosteus aculeatus* (Morris et al., 2014) and grass, *Panicum* (Lovell et al., 2016). In other cases, the heat sensitive population shows greater differential gene expression: copepod, *Tigriopus californicus* (Schoville et al., 2012), coral, *Acropora hyacinthus* (Barshis et al., 2013) and snail, *Chlorostoma funebralis* (Gleason & Burton, 2014). By comparing populations from the extreme ends of the distribution of *C. virginica*, we sought to test whether populations inhabiting divergent temperature regimes would mount the same transcriptomic response to changes in temperature ('general purpose' phenotype), and to what degree the response to temperature has diverged over the >18° of latitude and ~6000 km of coastline separating these two populations.

As with any trait, divergence in thermal physiology can be achieved through changes in gene regulation or through changes in protein coding sequences (Somero, 2010). Because RNA-seq data

TABLE 1 Summary results of prior physiological study (Casas et al., 2018). Per cent of time valve open represents the percentage of time that individuals were recorded with valves open, oxygen consumption rates were standardized by dry meat weight, to a standard oyster of 1 gram of dry meat weight, and clearance rate was expressed by gill area (cm^{-2})

	10°C	20°C	30°C
Percent of time valve open	64.4 ± 38.0%	91.0 ± 18.5%	61.4 ± 7.9%
Oxygen consumption rate (standardized by mean weight)	0.60 ± 0.28 mg O_2 hr^{-1} g^{-1}	0.99 ± 0.29 mg O_2 hr^{-1} g^{-1}	2.18 ± 0.67 mg O_2 hr^{-1} g^{-1}
Clearance rate (standardized by gill area)	0.11–0.34 Lh^{-1} cm^{-2}	1.04–2.52 Lh^{-1} cm^{-2}	1.36–2.87 Lh^{-1} cm^{-2}

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include both transcript abundances and nuclear DNA sequences of exons, it can be used not only to investigate divergence in gene expression between populations, but also whether some categories of genes show greater sequence divergence between populations, and whether divergence in gene expression corresponds to sequence divergence. Such interactions have been observed in a diverse and growing number of taxa: in the plant *Mimulus guttatus*, genes with elevated divergence in promoter regions between populations are disproportionately more likely to be differentially expressed between habitats (Gould et al., 2018), whereas in stickleback, genes that responded differentially to temperature between marine and freshwater populations are located within regions of genetic divergence implicated in local adaptation (Jones et al., 2012; Morris et al., 2014).

Whether or not divergent patterns of gene expression will be accompanied by observed divergence in mRNA sequences depends on the scale of linkage disequilibrium (LD). Divergence in gene expression may be caused by divergence in cis-regulatory sequences that create or disrupt transcription factor binding sites, altering baseline gene expression or its sensitivity to environmental cues (Wittkopp & Kalay, 2012). In these cases, we might expect to find elevated F_{ST} among these cis-regulatory sequences in comparisons between geographically isolated populations. Because RNA-seq data include only expressed sequences, we cannot directly observe divergence in these linked regulatory regions; however, depending on the scale of linkage disequilibrium, selection on regulatory regions may lead to regionally elevated F_{ST} values, extending into nearby coding regions. This will result in greater F_{ST} values for linked mRNA regions, but not elevated Ka/Ks ratios. The presence of both elevated F_{ST} values and Ka/Ks ratios would only be expected if selection were acting directly on protein coding sequences. On the other hand, if divergent gene expression occurs through changes in trans-regulatory sequences, or if the scale of LD is small, we might not observe elevated F_{ST} for genes that show divergent expression between populations. In light of this, we sought to directly test whether the convergence in phenotypes between these geographically isolated populations of *C. virginica* is a result of positive selection on gene expression or protein coding regions for transcripts that are differentially expressed in response to variation in temperature.

2 | METHODS

2.1 | Oyster collection and experimental conditions

Oysters were collected and experimentally manipulated as part of a prior research experiment (Casas et al., 2018). Briefly, wild oysters were collected from Baie Saint-Simon-Sud (47.7173°N; 64.7822°W), New Brunswick (NB), Canada and southeast Louisiana (LA), (Bay Gardene (29.5910°N; 89.6425°W) and Sister Lake (29.2341°N; 90.9172°W), USA. Oysters were acclimated to experimental temperature (10, 20 and 30℃) and salinity (15 and 25 PSU) conditions as described by Casas et al. (2018). Oysters were maintained at the experimental salinity and temperature combination for four weeks prior to sampling at which point, one gill was removed from 4 oysters per treatment and frozen for gene expression analysis. For this current study, only samples at 25 psu were considered, with a total of 24 samples (4 oysters × 2 populations × 3 temperatures) chosen for transcriptome profiling.

2.2 | RNA extraction and library preparation

Total RNA was extracted from extracted gill tissue using the 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 RNA quality was checked on a 2% agarose gel. Messenger RNA (mRNA) was isolated from ~500 ng of total RNA per sample 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.). Library quality and concentrations were checked using an Agilent 2100 Bioanalyzer high-sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA). Library concentrations were quantified using the NEBNext Library Quant Kit Quick Protocol (New England Biolabs, Inc.) on a ViiA-7 Real-Time PCR System (Thermo Fisher Scientific), and the KAPA Library Quantification Kit Data Analysis Template (Kapa Biosystems, Inc., Wilmington, MA, USA) was used to equally pool all samples. Libraries were sequenced using 2 approaches. For libraries coming from the 30°C and 20°C treatments, 100 bp paired-end reads were sequenced, and for the 10°C treatments, 100 bp single-end reads were sequenced, all on a HiSeq 3000 at Iowa State University's DNA Sequencing Facility (Ames, IA, USA). Due to this approach confounding the 10°C treatment and potential lane effects, we have analysed the data for the 30°C and 20°C treatments separately from the 10°C treatment.

2.3 | Sequencing Statistics

Sequencing of the 16 paired-end libraries from the 20 and 30°C treatments resulted in 559.4 million reads. Filtering of these reads left an average of 34.8 million reads per sample (min = 15.9 million, max = 88.8 million reads). Mapping of these reads to the reference transcriptome resulted in an average unique mapping rate of 56.8% (min = 20.8%, max = 64.2%). There was a single sample (LA 30°C replicate 1) with a low mapping rate (20.8%) which was driven by a large abundance of ribosomal RNA in the RNA-seq library. This sample was removed from further analysis, and the new average unique mapping rate was 59.6% (min = 46.6%, max = 64.2%). Sequencing of the 8 *SE* libraries from the 10°C treatment resulted in 139.3 million reads. Filtering of these reads left an average of 17.4 million reads per sample (min = 12.9 million, max = 21.5 million reads) with an average

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unique mapping rate of 59.1% (min = 53.4%, max = 62.4%). The final overall mapping rate of the 23 remaining libraries was 59.3% (min = 46.6%, max = 64.2%) with an average of 21.4 million reads per sample distributed across 39,494 protein coding sequences (genes). Mapping rates per population were also similar, mean mapping rate for LA and NB were 58.2% and 60%, respectively. Filtering of the count data to remove lowly expressed genes reduced the data set down to 36,479 CDS regions (genes) retained for differential expression analysis.

Whenever reads from two or more populations are mapped to a common reference, observations of differential gene expression may be confounded by elevated divergence in some genes. This will cause problems if divergence causes one population's reads to map less efficiently, creating the appearance of lower expression for the more divergent genes. To test for this problem in our data, we calculated pi for each gene relative to the reference for each gene in each population. We then tested for a relationship between difference in divergence from the reference (piNB–piLA) versus observed difference in expression (CPM_NB-CPM_LA; CPM = counte per million). If divergence has no influence on mapping rates, then there will be no relationship between divergence from the reference and observed difference in expression. However, if the population with the more diverged allele has a lower mapping rate, then we expect a negative relationship, with increased divergence from the reference resulting in lower observed expression.

2.4 | Calling SNP variants from the RNA-seq reads

The RNA sequencing reads were also used to identify single nucleotide polymorphisms (SNPs) between the two populations. For this analysis, we only used the subset samples for which we had paired-end sequencing reads and followed the GATK-best practices workflow for SNP and indel calling on RNA-seq data (https://software.broadinstitute.org/gatk/best-practices/). The paired-end reads were mapped to the published genome using the sequence aligner STAR (v.2.7.1), employing a 2-pass approach to account for both annotated and unannotated splice junctions. The aligned reads were converted to BAM files and read groups added in order to account for variation associated with population, treatment and HiSeq sequencing lane. Read alignments were deduplicated and indexed using Picard tools (v.2.0.1). Deduplicated sequence reads were split into exons and hard-clipped to remove reads covering introns using gatk (v.4.0.2.1). These exon-specific sequences were used to call variants with the GATK function HaplotypeCaller with a stand-call-conf of 20.0 and excluded soft-clipped-bases. All identified variants were merged using the GATK function CombineGVCFs, and this master file of variants was used to mask potential SNPs during base recalibration. Base recalibration was completed twice on each sample, first with masking potential SNPs and a second pass with a sample-specific recalibration table generated from the first pass and completed using the GATK function ApplyBQSR. Variants were again called against the reference genome using the now re-calibrated, deduplicated, exon-specific sequence reads with HaplotypeCaller allowing for soft-clipped-bases and again setting the stand-call-conf to 20.0. Variants were hard filtered with the GATK function VariantFiltration with a window size of 35 and cluster = 3, as suggested in the RNA-seq best practices workflow. Filtered variants were merged using CombineGVCFs, and the combined VCF file was filtered using VCF tools with settings requiring the SNP to be present in 50% of samples per population, with a minimum quality score of 30, minimum coverage of 10, a minor allele frequency of 0.05 and a max allele frequency of 0.95. Weighted estimates of F_{ST} were subsequently calculated across a 1000-bp sliding window with a 1000-bp step size using VCFtools (v.0.1.14), and gene-wide mean weighted F_{ST} values were calculated by calculating the mean weighted F_{ST} of every window overlapping a given gene feature. Finally, we used the python-based program SNPeff (v.4.3T) to obtain an estimate of Ka/Ks and annotation of effect for each SNP based on where within the gene the polymorphism occurs. The Ka/Ks data set was filtered to remove genes from the analysis for which greater than 95% of variants were identified as nonsynonymous mutations. This was done in order to avoid comparing paralogs between these two populations. For both F_{ST} and Ka/Ks analyses, functional enrichment was conducted using only genes that passed both F_{ST} and Ka/Ks filtering with Fisher's exact test to explore gene ontology enrichment within the genes that had F_{ST} values above the 95% quantile (F_{ST} = >0.392) or a Ka/Ks ratio >1.

2.5 | Differential gene expression analysis

For differential expression analysis, the forward read from each paired-end library and all single-end sequence reads were trimmed of adapters and low-quality bases using Trimmomatic (v.0.38) (Bolger, Lohse, and Usadel, 2014) with the following parameters (PE data: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 and the single-end data: ILLUMINACLIP:TruSeq3-SE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36), and sequencing quality checked using the software FastQC (v.0.11.5) (Andrews, 2010). Trimmed reads were mapped to gene coding sequences (CDS) annotated in a haplotig-filtered reference genome for *C. virginica* (GCF002022765.2), using bowtie2 (Langmead & Salzberg, 2012) as implemented in RSEM (v.1.3.1) and differential expression measured using R (v.3.5.1) with the package edgeR (v.3.22.5). For these analyses, two separate counts matrixes were generated, one for the PE data and one for the SE data. For each group, reads were filtered using a cut-off of 0.5 count per million in at least 4 samples, samples were normalized with post-filtering library sizes, and a negative binomial generalized log-linear model was fit using the glmFit function in edgeR. Global patterns in gene expression were analysed with a principal coordinate analysis (PCoA) using log-transformed reads generated using the cpm() function in edgeR with log = T and prior.count = 2. These log-transformed reads were used to calculate dissimilarity indices using the R program vegan (v.2.5-5) and the pcoa function from the R program ape (v.5.3). The influence of

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temperature and population was analysed on the log-transformed reads from the 20°C and 30°C treatment samples only, using the adonis2 permutational multivariate analysis of variance with the formula accounting for temperature, population and temperature-by-population effects (Pop + Temp + Pop*Temp) in the R program vegan with 1,000,000 permutations. The influence of population was also analysed for the 10°C treatment samples using 1,000,000 permutations.

Differential expression (DE) analysis between treatments was tested using a combination of pairwise assessments of expression. For these analyses, we considered baseline population differences in expression by testing for pairwise DE between populations at each temperature (e.g. NB 30°C–LA 30°C, NB 20°C–LA 20°C, and NB 10°C–LA 10°C), and results from this analysis can identify baseline differences in gene expression with LA as the reference population. We also tested for the main effect of temperature between the 30°C and 20°C treatments that were sequenced together, with the two populations considered independently, and results from this analysis are able to identify differential expression between temperature treatments for each populations (e.g. LA 30°C–LA 20°C and NB 30°C–NB 20°C). For each of these comparisons, we tested for functional enrichment based on the signed-log *p*-value of the transcriptome-wide (i.e. all genes in the measured transcriptomes: 20°C and 30° comparisons $n = 32{,}542$; 10°C comparisons $n = 26{,}287$) expression results. Enrichment was tested across all 3 major gene ontologies (i.e. biological process (BP), cellular component (CC) and molecular function (MF)) with a Mann–Whitney U (MWU) test in R using the package ape (v.5.2) and code developed by Wright, Aglyamova, Meyer, and Matz (2015).

We also identified overlap in the DEGS from the temperature and population pairwise comparisons to identify (1) genes differentially expressed only between populations, (2) genes differentially expressed only between temperatures and (3) genes differentially expressed between both populations and temperatures. The genes in group 1 are those that appear to have diverged in expression between populations but are not significantly DE between 20 and 30°C. The genes in group 2 are those that are differentially expressed between temperatures, but not differentially expressed between populations. And the genes in group 3 are those that are differentially expressed between populations and between the two warmer temperature treatments. Hierarchical clustering of each of these groups was then used to identify the subsets of genes predominantly up-regulated and those predominantly down-regulated. Each of these gene clusters was also tested for functional enrichment of GO terms using Fisher's exact test. Note this value represents only genes that were included in both the 10°C treatment and 20 and 30°C treatments ($n = 25{,}609$). All functional enrichment results are available in Table S1 and File S1.

Finally, in order to test whether differentially regulated genes had elevated F_{ST} or Ka/Ks ratio, we used a series of permutation tests to compare the mean of the DEGs against the genome-wide average. For this analysis, the mean F_{ST} (or Ka/Ks ratio) was calculated for each of these 3 groups of genes. Then, using the same number of significant genes, the F_{ST} (or Ka/Ks ratio) values were shuffled in R using the sample function to generate a new difference in means and this was repeated 1,000,000 times to generate a null distribution. This allowed us to test whether the mean difference in the DE genes was significantly different than the mean difference in the permuted list. Finally, by taking the mean of the absolute value of the permuted list, we were able to calculate a *p*-value based on the number of times the permuted value was greater/less than the observed value.

3 | RESULTS

3.1 | Nucleotide diversity versus expression

We tested for a relationship between divergence from reference for each population and considered how the difference between divergence for each population (pi_{NB}–pi_{LA}) relates to the difference in normalized expression between populations (CPM_{NB}–CPM_{LA}). This analysis showed that for the transcriptome as a whole, there was no relationship between nucleotide diversity gene expression. However, our analysis identified a single outlier: a heat shock 70 kDa protein 12B-like gene (XP_022324405.1) which was found to be up-regulated by fivefold in NB compared to LA at every temperature but was not differentially expressed between temperatures in either population.

3.2 | Population differences in F_{ST}

Sequence divergence analysis between the New Brunswick and Louisiana populations was summarized across a reduced set of 16,814 genes. These genes were found to have a mean weighted F_{ST} of 0.152 ± 0.12. We explored functional enrichment among the genes that showed an elevated F_{ST} (defined as genes in the 90th percentile, $F_{ST} > 0.313$) using Fisher's exact test. This analysis included 1,682 genes with an elevated F_{ST} and was tested against the background list of 16,814 genes. Functional enrichment of these genes identified 8 enriched ontologies. The enriched ontologies included a *growth factor activity* where 8 of the 15 associated genes were found to be elevated in F_{ST}. There was also enrichment for *cation transport*, *ion transmembrane transporter activity* and *receptor regulator activity* (Table S1). These terms were found to be highly related forming two clusters of ontologies associated with either transmembrane transport or growth and carbohydrate biosynthesis (Figure S1a-b).

3.3 | Population differences in Ka/Ks ratio

In addition to F_{ST}, we examined sequence divergence between the NB and LA populations by comparing the rate of synonymous versus nonsynonymous mutations within coding regions of the 16,814 genes used in the F_{ST} analysis. This analysis found that 4,415 of the 16,814 genes had a Ka/Ks ratio >1. Fisher's exact test found significant enrichment for 61 gene ontology (GO) terms including 6 ontologies associated with peptidase or hydrolase activity (Table S1). Of these, the 16

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genes associated with the GO term, *cysteine-type endopeptidase activity involved in apoptotic process*, were all found to have an elevated Ka/Ks ratio. In addition, the enriched GO term *lipopolysaccharide receptor activity* was found to have 8 genes with elevated Ka/Ks ratio out of the 10 genes associated with the term. Clustering of these GO terms highlights that immune system or defence related ontologies represent nearly half of all enriched terms (Figure S1c–e). Interestingly, there was no overlap between these enriched ontologies for Ka/Ks ratios and those that were enriched among genes with elevated F_{ST}.

3.4 | Global Patterns of expression

Global patterns in gene expression between the genes passing expression threshold filtering were explored using a PCoA for each sequencing group independently (Figure 1a, b). The permanova performed with the log_2-transformed counts for the 20°C and 30°C treatments identified a significant influence of population (p-value = 1e−06), temperature (p-value = .009), but not the interaction of temperature by population (p-value = .244), and a significant influence of population for the 10°C treatment (p-value = .028). In total, the first two principal components accounted for 28% and 41% of the transcriptomic variation in the warm and cold treatments, respectively, and revealed clear separation between both populations at each temperature.

3.5 | Comparison 1: Population differences in expression at each temperature

The pairwise assessments of gene expression between populations showed significant levels of DE for each treatment with 698 DEGs at 10°C, 480 DEGs at 20°C and 638 DEGs at 30°C. Functional enrichment of each of these sets of genes found 101 enriched gene ontologies in the 10°C treatment, 69 enriched ontologies at 20°C and 13 enriched ontologies at 30°C. Surprisingly, 75% of these enriched ontologies were found to be down-regulated in the NB population. The majority of these enriched ontologies were differentially expressed in the same direction with the exception of *Hydrolase activity, G-protein-coupled receptor activity and Molecular transducer activity* (Table 2).

3.6 | Comparison 2: Genes showing main effect of population

When considered together, the 3 pairwise assessments of differential gene expression between populations identified a total of 1,228 DEGs. Of these, only 998 genes were not also DE between temperatures. These 998 population genes were found to group into 2 clusters of expressed genes: (population cluster 1) those predominately down-regulated in NB and (population cluster 2) those predominately up-regulated in NB when compared to LA oysters at each temperature (Figure 2—Population Heat map). We tested for GO enrichment for each of these two subgroups using Fisher's exact test that found significant enrichment for 4 MF and 2 BP terms among the genes down-regulated in NB (Table S1). The enriched terms included *superoxide metabolic process*, *signal receptor binding* and *dipeptidyl peptidase*. The genes in population cluster two in contrast were enriched for a single ontology, *S-methyltransferase activity* (p-value = .007). These genes (both cluster 1 and 2) were found to be significantly elevated in both F_{ST} and KaKs metrics when compared to the transcriptome-wide average (Figure 2).

3.7 | Comparison 3: Genes showing main effect of temperature

The pairwise test for genes showing a main effect temperature identified a total of 650 genes that were DE between 30°C and 20°C

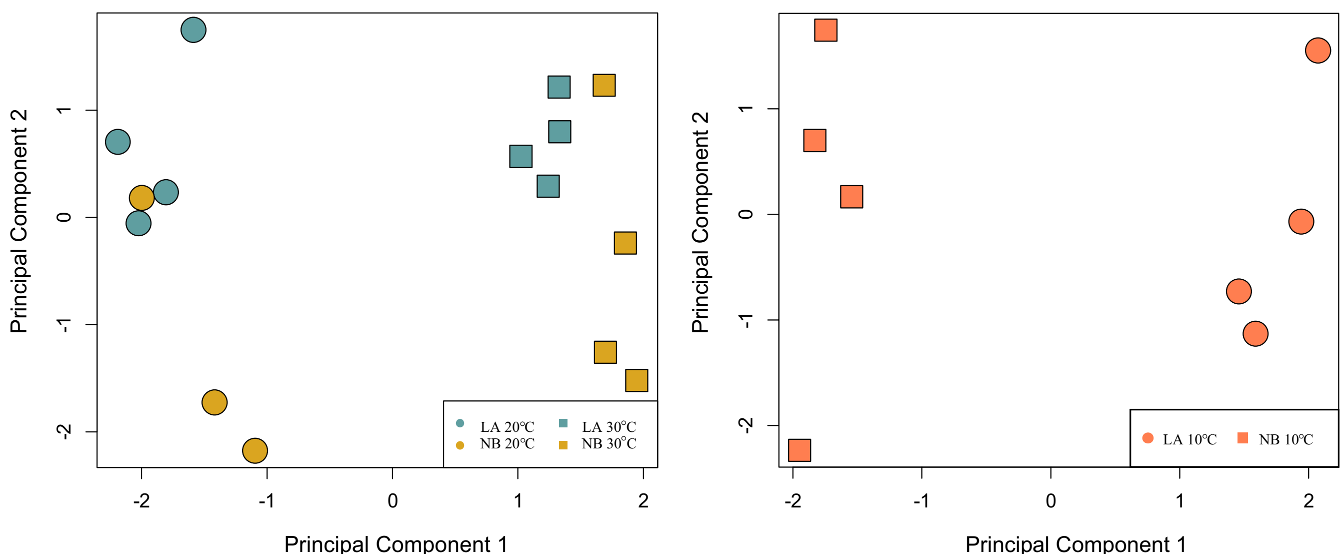

FIGURE 1 PCA analysis with grouping by treatment

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TABLE 2 GO enrichment for population differential expression

GO Category	GO Ontology	GO IDs	# seqs (% of ref)	30°C Adjusted *p*-value	20°C Adjusted *p*-value	10°C Adjusted *p*-value
Molecular function	Ionotropic glutamate receptor activity	GO:0004970 GO:0008066	17 −48.50%	5.50E-03	2.47E-02	–
	ATPase activity, coupled to movement of substances	GO:0042626 GO:0015405 GO:0043492 GO:0015399	48 −31.80%	5.20E-03	2.99E-03	–
	Enzyme regulator activity	GO:0030234	91 −29.40%	–	9.32E-03	–
	Hydrolase activity, acting on acid anhydrides	GO:0017111 GO:0016462 GO:0016818 GO:0016817	40 −27.00%	–	7.73E-02	5.50E-02
	Enzyme binding	GO:0019899 GO:0051020	71 −28.20%	–	2.47E-02	1.08E-03
	Protein serine/threonine kinase activity	GO:0004674	46 −27.80%	–	7.19E-02	3.41E-02
	NADH dehydrogenase (ubiquinone) activity	GO:0008137 GO:0050136 GO:0003954 GO:0016655	7 −70%	–	3.28E-02	4.71E-02
	Molecular transducer activity	GO:0004888 GO:0038023 GO:0060089		–	9.32E-03	4.86E-02
	G protein-coupled receptor activity	GO:0004930	185 −30.70%	–	7.37E-03	4.86E-02
Biological process	Organonitrogen compound biosynthetic process	GO:1901566	119 −25.40%	3.08E-02	5.74E-04	–
	Immune system process	GO:0002376	48 −37.20%	–	9.62E-03	–
	Electron transport chain	GO:0022900 GO:0022904	3 −10%	–	2.04E-03	–
	Protein folding	GO:0006457	29 −47.50%	–	–	3.47E-03
	Immune response	GO:0006955	28 −37.30%	–	1.77E-02	8.52E-02
	Energy coupled proton transport	GO:0015986 GO:0015985	9 −90%	–	5.74E-04	2.92E-02
Cellular component	Proton-transporting ATP synthase complex, coregulatedling factor f(o)	GO:004526	7 −46.60%	–	3.56E-02	4.76E-02
	Oxidoreductase complex	GO:199020	6 −18.20%	–	4.55E-02	4.69E-03
	Mitochondrial membrane part	GO:009880 GO:004445	12 −20.70%	–	3.75E-03	3.71E-04

Note: Blue cells represent down-regulated ontologies in the NB population compared to LA, and red cells represent up-regulated ontologies in the NB population compared to LA for each temperature. Adjusted p-value is from the Mann–Whitney U test.

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in either of the populations. There were only 292 genes that were found to not also be DE between population (Figure 3). Clustering of these genes into 2 clusters showed that the logFC of these genes was always stronger in NB. This was seen when comparing the mean logFC in expression which was found to be on average 2.4 times higher in the NB population (mean logFC: NB +2.53; mean logFC LA +1.06). Likewise, the down-regulated genes were on average 1.4 times lower in measured logFC in the NB population (mean logFC: NB −3.12; LA −2.18). Neither cluster of genes was found to be enriched for any ontological function; however, considering all 292 DEGSs together did identify enrichment of a single GO term; *clathrin coat* (*p*-value = .02). Finally, this set of genes was found to have a significantly elevated F_{ST}; however, the mean KaKs ratio was not significantly different from the global mean (Figure 3).

3.8 | Comparison 3: Genes showing shared effects of temperature and population

Intersecting the population and temperature results identified 247 genes showing both temperature and population effects. These genes were found to group into 4 clusters of genes showing both temperature and population level differences (Figure 4). Functional enrichment testing of genes in cluster 1 identified no enrichment. Cluster 2 genes showed enrichment for 4 related GO terms associated with extracellular matrix proteins. Cluster 3 had enrichment for 2 MF ontologies associated with oxidoreductase activity, and cluster 4 had no significant enrichment of any GO terms. The genes within these two clusters that show functional enrichment (Clusters 2 and 3) highlight an interesting pattern where the strongest level of differential expression is observed again in the NB population. These genes were also found to have elevated F_{ST} and KaKs ratios similar to what was observed for the population only genes.

4 | DISCUSSION

In this study, we sought to test whether the wide-ranging eurythermal invertebrate *Crassostrea virginica* has achieved its broad distribution through divergent transcriptomic responses to temperature. We did this by comparing gene expression across 10, 20 and 30°C acclimation temperatures for two populations at the extreme ends of this species' geographic range. We observed a core set of genes with similar responses to temperature, but also a large number of genes with expression responses to temperature that were unique to each population. When considering the differences between the two warmer temperatures, we observed a larger gene expression response in the New Brunswick population, which has had less historical exposure to heat stress. A similar pattern between populations with differing historical exposures to stress has also been observed in *Acropora hyacinthus* corals (Barshis et al., 2013) and *Chlorostoma funebralis* snails (Gleason & Burton, 2014). On the other hand, in *Porites astreoides* corals, elevated plasticity of gene expression in response to heat stress is instead associated with greater stress

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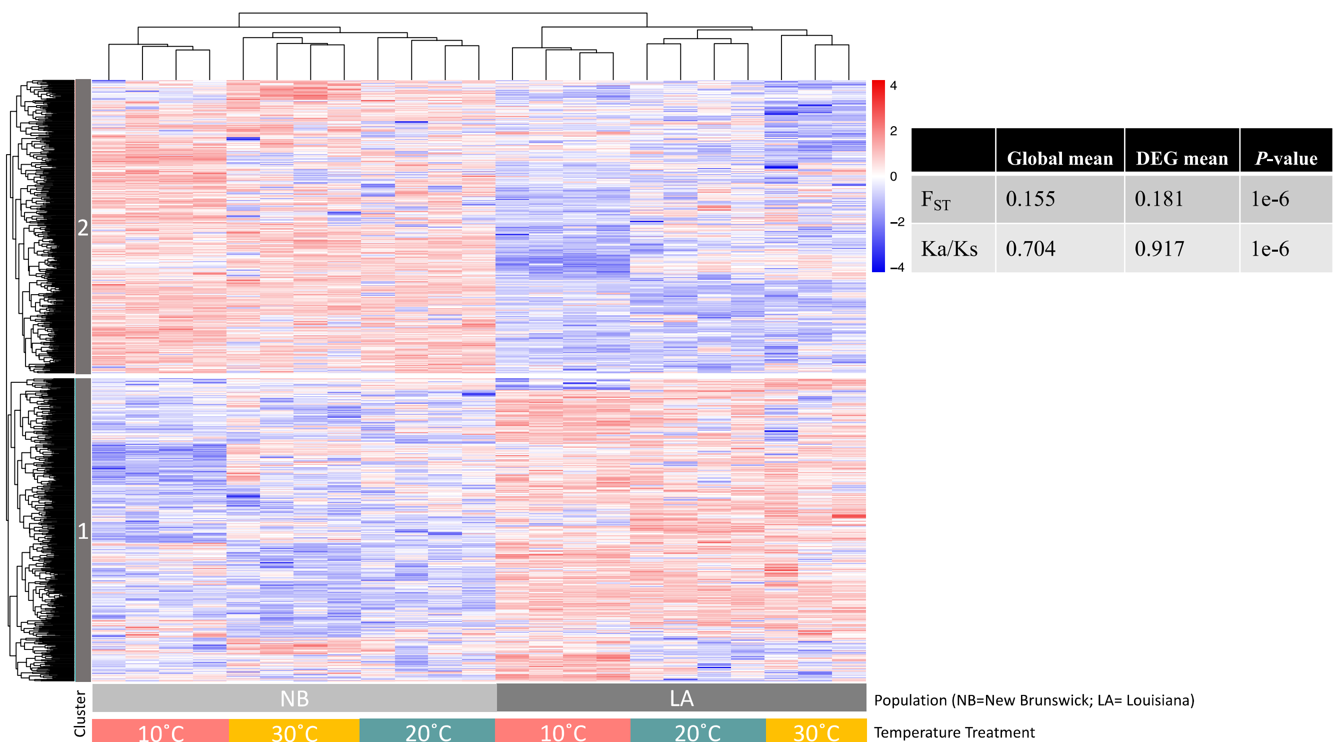
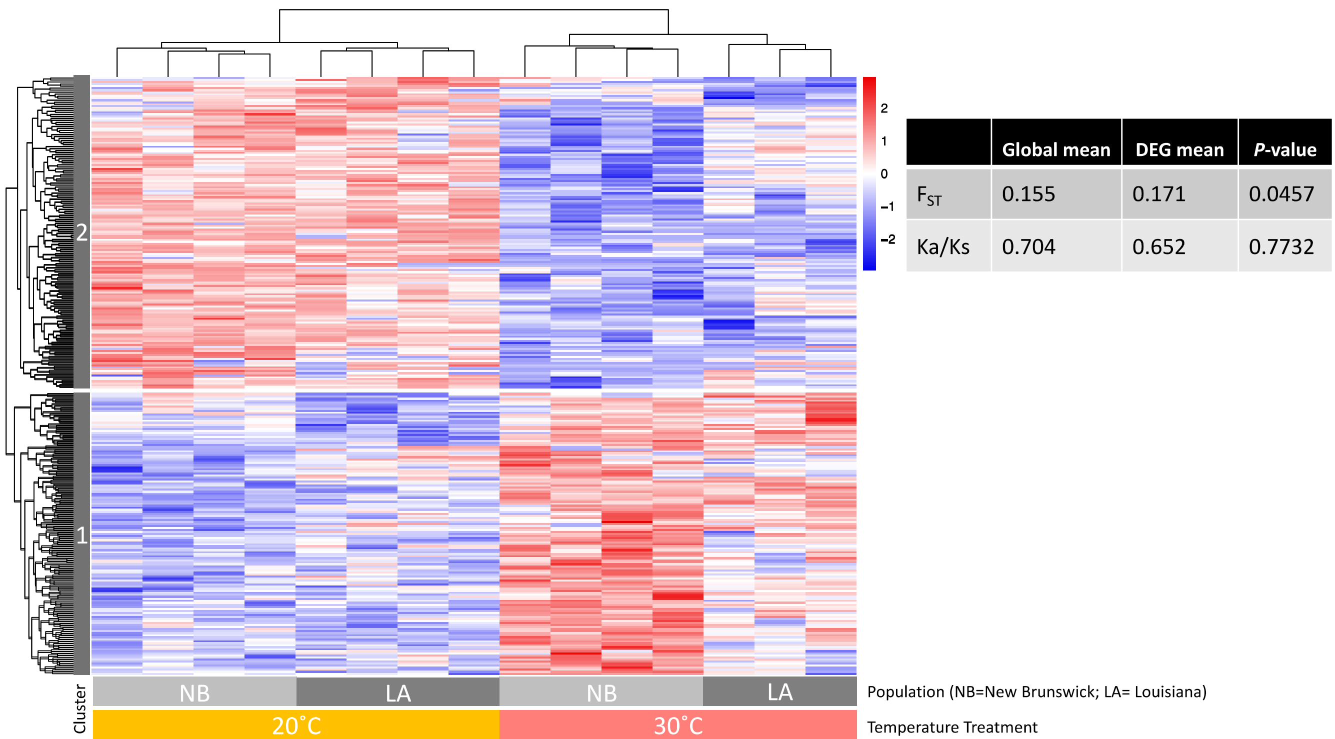


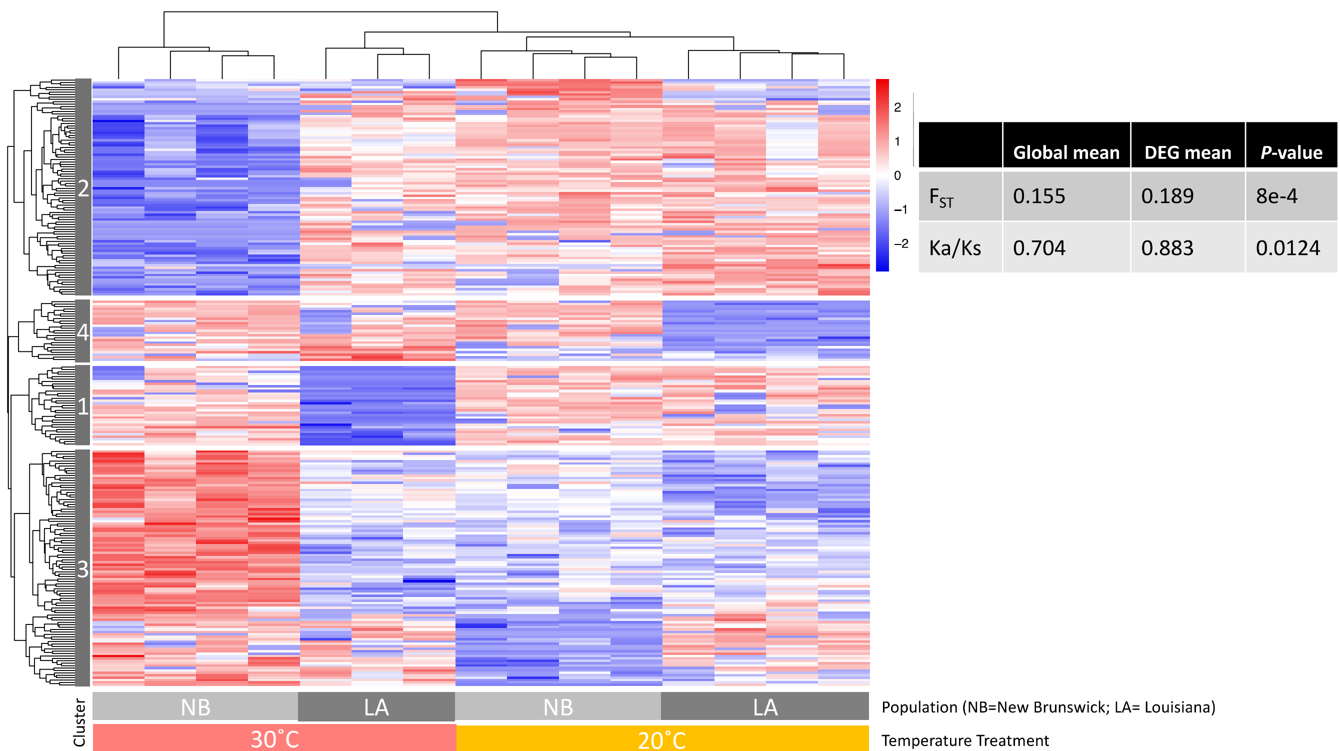
	Global mean	DEG mean	*P*-value
F_{ST}	0.155	0.181	1e-6
Ka/Ks	0.704	0.917	1e-6

FIGURE 2 Heatmap of all differentially expressed genes with a main effect of population (*n* = 998). Gene clusters 1 and 2 are highlighted with grey bars. Adjoining table presents transcriptome-wide "Global" mean and the reduced differentially expressed gene set's mean F_{ST} and Ka/Ks values. *p*-Values report significance from permutation test with $1E^6$ permutations



	Global mean	DEG mean	*P*-value
F_{ST}	0.155	0.171	0.0457
Ka/Ks	0.704	0.652	0.7732

FIGURE 3 Heatmap of all differentially expressed genes with a main effect of temperature ($n = 292$). Gene clusters 1 and 2 are highlighted with grey bars. Adjoining table presents transcriptome-wide "Global" mean and the reduced differentially expressed gene set's mean F_{ST} and Ka/Ks values. *p*-Values report significance from permutation test with $1E^6$ permutations



	Global mean	DEG mean	*P*-value
F_{ST}	0.155	0.189	8e-4
Ka/Ks	0.704	0.883	0.0124

FIGURE 4 Heatmap plotting of all differentially expressed genes in both the temperature and population comparisons ($n = 247$). Gene clusters 1,2,3 and 4 are highlighted with grey bars. Adjoining table presents transcriptome-wide "Global" mean and the reduced differentially expressed gene set's mean F_{ST} and Ka/Ks values. *p*-Values report significance from permutation test with $1E^6$ permutations

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tolerance (Kenkel & Matz, 2017). As in several other studies (Barshis et al., 2013; Gleason & Burton, 2014; Schoville et al., 2012), we also observed minimal overlap between genes differentially expressed between temperatures and between populations.

Because animals used in this experiment were collected from wild populations, variation in gene expression may also have arisen from the differences in the history of each population. In particular, differences in oyster ages may have an impact on gene expression. In the Pearl oyster, age had a significant influence on the expression of immune and biomineralization genes following a nucleus insertion operation used by pearl-producing operations (Adzigbli et al., 2019). In addition to age, the difference in the environments between the two collections sites may be producing an epigenetic effect on gene expression (see reviews, Eirin-Lopez & Putnam, 2019; Hofmann, 2017). However, although there may be these additional explanations for differences in gene expression between populations (e.g. epigenetic effects, transcriptomic frontloading), our results comparing F_{ST} and Ka/Ks ratios between DE groups provide evidence that some of the differences in expression between populations reflects selection on gene expression.

Each of the three temperature comparisons provides unique insights into how eastern oysters have adapted to the contrasting environments of Louisiana and New Brunswick. We would predict that oysters originating from NB may harbour adaptations important for survival in low temperatures although LA oysters may harbour adaptations important for survival at elevated temperatures. As such, the enrichment of up-regulated GO terms at 10°C shows that the NB population was maintaining enzyme function and cellular signalling, suggesting that NB maintained critical cellular process at low temperatures that were suppressed in the LA population. These responses were coupled with a greater magnitude of down-regulated GO terms possibly involved in growth and transmembrane signalling including *carbonate dehydratase* and the broad *structural molecule* term indicating a shift to maximize growth rate in warmer temperatures for the more southern population. Similar Gene Ontology enrichments have been seen in the Pacific oyster (*C.gigas*) where individuals bred for faster growth rates were also characterized in part by decreased expression of genes involved in the phosphatidylinositol signalling system and calcium signalling (Zhang et al., 2019).

Likewise, we expected that oysters conditioned to 20°C would show minimal differences between the two populations as this temperature is well within the annual range of temperatures that both populations regularly experience. As such it was surprising to see significant down-regulation in NB of GO terms associated with multiple forms of transmembrane transport (*sodium ion transmembrane transporter*, *ion transmembrane transport*, *cation channel*, *cation transmembrane transport*, *ligand-gated channel*, *ionotropic glutamate receptor* and the broad *structural molecule* term). The observation that the NB population down-regulates transmembrane transporters and the continued down-regulation of *structural molecule* suggests that key population differences may be associated with transmembrane ion transport and structural composition of the cellular membranes at low temperatures. There are multiple potential drivers behind these responses including (a) the morphology of these organisms as the NB oysters were found to have increased gill surface area in comparison with the oysters from LA (Casas et al., 2018). This increase in gill may be an adaptation in the northern population to extract the most out of the environment in response to short growing seasons, and as a result may require a larger transcriptomic signature of this commonly observed cellular response to altered temperature, membrane remodelling (Hazel, 1995; Hazel & Williams, 1990). Additional factors influencing the observed response include (b) that adaptation to low salinity conditions in the LA population has resulted in constitutively higher expression of ion transporters or (c) that the environmental history differences between the individuals used in this study have distinct environmentally induced epigenetic profiles driving differences in constitutive expression.

Of interest too is that at the high temperature treatment (30°C), the enriched terms between populations were an up-regulation of ATPase activity, associated with a broad range of molecular processes, and down-regulation of ionotropic glutamate receptor activity and carbohydrate derivative biosynthetic processes suggesting that even at this high temperature, a major difference between the two populations is associated with an increased expenditure of ATP and a decrease in processing of sugars. This decrease in expression of genes associated with processing of carbohydrates among the NB individuals is interesting, because although there were no observed differences in feeding rates between the two populations at any temperature, the LA oysters were found to have a better overall condition index and a lower overall respiration rate and metabolic activity, suggesting that this down-regulation may be a signature of NB oysters running out of energetic reserves (Casas et al., 2018). It is possible that this difference is also explained by the overall lower rate of respiration among the LA individuals regardless of what temperature/salinity combination they were exposed to (Casas et al., 2018). Additional evidence for intraspecific variation in growth along latitudinal gradients has been found in many ectotherm species, with higher rates at higher latitudes (Lindgren & Laurila, 2009). As organisms from higher latitudes have a restricted time available for growth, it is possible that the oysters from NB have adapted more efficient uses of their energy reserves in response to a seasonal reduction in resource availability.

Our analysis indicates that genes that were differentially expressed by population have elevated F_{ST} values and elevated Ka/Ks values relative to the genome-wide average. This included genes that were differentially expressed by population only (Figure 2) and genes that were differentially expressed by population and by temperature (Figure 4). By contrast, genes that were differentially expressed by temperature only showed only marginally elevated F_{ST} values and Ka/Ks values that were no different from the genome-wide average (Figure 3). Expression divergence is correlated with sequence divergence among populations in plants (Gould et al., 2018), stickleback (Jones et al., 2012; Morris et al., 2014) and mice (Giorello et al., 2018). In *Arabidopsis*, stress responsive genes had lower Ka/Ks ratios, elevated F_{ST} and elevated nucleotide diversity in promoters in comparisons between populations, all suggestive of a prominent

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role for cis-regulatory divergence in shaping local adaptation (Lasky et al., 2014). We expect that elevated F_{ST} values in genes that were differentially expressed between populations in this study stem from selection on linked cis-regulatory regions; however, because Ka/Ks values were also elevated in this set of genes, we cannot exclude the possibility that that elevated F_{ST} stem from selection on protein coding regions themselves. Our observation that Ka/Ks values were elevated in genes that were differentially expressed between populations is also consistent with results from previous studies. Many taxa show a negative relationship between gene expression and Ka/Ks values, with more highly expressed housekeeping genes often being the most conserved, and thus subject to the strongest purifying selection (Pal et al., 2001; Subramanian & Kumar, 2004; Wright et al., 2004). On the other hand, elevated amino acid sequence divergence may correlate with expression divergence (Nuzhdin et al., 2004). Although we observed Ka/Ks values for DE genes that were elevated relative to the genome as a whole, the mean Ka/Ks for these genes is still less than 1, meaning that on average, these genes are still subject to purifying selection. It is possible that that elevated Ka/Ks for these genes represents positive selection on some genes within the set. Alternatively, it is possible that genes that are differentially expressed between populations are simply less conserved and thus experience relaxed purifying selection relative to the genome as whole. The fact that genes responding to temperature alone did not show elevated Ka/Ks values is consistent with results from *Daphnia*, where genes implicated in thermal tolerance did not show elevated coding sequence divergence among thermally adapted populations, and there was little overall relationship between genes showing expression divergence between populations and genes showing coding sequence divergence (Herrmann et al., 2018).

We observed only 10 annotated transcripts that were differentially expressed between temperature comparisons that also had an elevated F_{ST} including a heat shock protein 27-like gene (F_{ST} 0.44; Ka/KS 0.94). Similar hsp-27 like genes have also been seen to be DE in a previous transcriptome study using selectively bred *C. gigas* oysters known to differ in the degree of heat shock tolerances. In that study, Lang et al. (2009) found that hsp-27 expression was higher in temperature sensitive population after 6 hr of exposure; however, the observed increase in expression disappeared after 24 hr of sampling. In this study, hsp-27 expression was lowest in the NB 30°C treatment (12 cpm) being significantly lower in expression than either the 20°C (650 cpm) or 10°C (1,174 cpm) treatments. This is in contrast to no differential expression of hsp-27 in the LA population where expression was not variable between temperatures and was half level as was observed for the NB population at the 20°C treatment. These results suggest that the NB population maybe more sensitive to increased temperatures compared to the LA population, based on the ability of NB oysters to dynamically vary the expression of hsp-27, a trait Lang et al.,(2009) associated with temperature sensitivity. There was also significant differential expression of a MAM and LDL-receptor class A domain-containing protein 1-like gene that showed significant elevated F_{ST} (0.43) and Ka/Ks ratio (1.38) in addition to being differentially expressed between temperatures and populations with increased expression in the LA population occurring only at 30°C whereas the NB population increased expression at the 20°C treatment. This pattern of up-regulation of a peptidase coding gene showing signatures of selection suggests that adaptation to distinct temperature regimes may have influenced metabolic processes at lower temperatures in NB compared to LA.

These patterns also have implications for understanding how oysters in the New Brunswick population will respond to warming ocean conditions. The combined observations of limited physiological differences in response to temperature (Casas et al., 2018), with a largely divergent transcriptomic responses to temperature, suggest there has been some degree of local adaptation that has occurred between oysters from these two regions. However, the 1,228 genes that are differentially expressed in response to temperature show a signal of selection on genomic sequences (F_{ST}) but not amino acid (Ka/Ks ratio; Figure 3) suggesting that ocean warming in the North Atlantic Ocean will not likely have a large negative effect on oyster thermal physiology. There still remains, however, the potential for additional stressors to develop alongside ocean warming (e.g. deoxygenation, changes to salinity, increased disease prevalence and acidification) that may further negatively impact these populations (Claret et al., 2018, Burge et al., 2014, Jones et al., 2019). In addition, although this study was an extended acclimation study, it is unable to determine whether the increased metabolic rate of New Brunswick oysters will have long-term negative impacts on survival or fecundity. Future studies examining the impact of warming, hypoxia and infection would add valuable insights into the resiliency of these northern-most populations of *C. virginica*.

In all, our analysis has highlighted that temperature responses between populations have diverged at the transcriptome level whereas maintaining a similar yet shifted physiological response. Our analysis identified significant divergence in F_{ST} but not amino acid sequences with genes that exhibit differential expression between the two extreme temperatures. This increase in F_{ST} is likely attributable to selection on cis-regulatory regions. In contrast, the significant divergence in Ka/Ks ratio and F_{ST} for genes differentially expressed between populations may be experiencing selection on both protein coding and gene regulatory regions; however, the low overall average of these ratios still reflects purifying selection (Ka/Ks < 1). Together, our findings suggest that genetic variation associated with adaptation to temperature and the maintenance of physiological functions in eastern oysters is likely driven by selection on gene regulatory regions but not protein coding changes.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The RNA-seq data presented here are available through the NCBI SRA database under BioProject accession PRJNA719567. All associated codes for the analysis are included in the supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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