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Lack of genotype-by-environment interaction suggests limited potential for evolutionary changes in plasticity in the eastern oyster, *Crassostrea virginica*

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Abstract

Eastern oysters in the northern Gulf of Mexico are facing rapid environmental changes and can respond to this change via plasticity or evolution. Plasticity can act as an immediate buffer against environmental change, but this buffering could impact the organism's ability to evolve in subsequent generations. While plasticity and evolution are not mutually exclusive, the relative contribution and interaction between them remains unclear. In this study, we investigate the roles of plastic and evolved responses to environmental variation and *Perkinsus marinus* infection in *Crassostrea virginica* by using a common garden experiment with 80 oysters from six families outplanted at two field sites naturally differing in salinity. We use growth data, *P. marinus* infection intensities, 3′ RNA sequencing (TagSeq) and low-coverage whole-genome sequencing to identify the effect of genotype, environment and genotype-by-environment interaction on the oyster's response to site. As one of first studies to characterize the joint effects of genotype and environment on transcriptomic and morphological profiles in a natural setting, we demonstrate that *C. virginica* has a highly plastic response to environment and that this response is parallel among genotypes. We also find that genes responding to genotype have distinct and opposing profiles compared to genes responding to environment with regard to expression levels, Ka/Ks ratios and nucleotide diversity. Our findings suggest that *C. virginica* may be able to buffer the immediate impacts of future environmental changes by altering gene expression and physiology, but the lack of genetic variation in plasticity suggests limited capacity for evolved responses.

KEYWORDS
common garden, evolution, genotype-by-environment interaction, plasticity, transcriptomics

1 | INTRODUCTION

Organisms can respond to environmental change through plasticity or evolution (Hoffmann & Sgro, 2011; Parmesan, 2006; Seebacher et al., 2015). Plasticity describes the variation in phenotypes produced from a single genotype within a single generation (DeWitt & Scheiner, 2004; West-Eberhard, 2003). This variation can act as an immediate buffer against environmental challenges, although it is not expected to produce additional change over successive generations beyond the range achieved in the initial response (Seebacher et al., 2015). In contrast, evolution is a multigenerational process by which allele frequencies of a population change over time as a

result of differential survival and reproductive success among genotypes. Although plasticity and evolution are not mutually exclusive (plasticity itself can evolve, Via & Lande, 1985), their relative contributions and the interactions between them in populations responding to environmental change remains unclear (Forsman, 2015; Ghalamnor et al., 2007; Hendry, 2016; Jong, 2005; Price et al., 2003; Wund, 2012).

One approach to quantifying the relative contributions of plasticity and genetic variation to phenotypic responses to environmental change is comparative transcriptomics (DeBiasse & Kelly, 2016). Transcriptomics can be used to better understand plastic and evolutionary responses to changing environments because transcriptomes, along with physiological traits, are phenotypes resulting from the combination of the genotype (G, genetic variation in expression), environment (E, plasticity, the environmental effect on expression) and genotype-by-environment interaction (GxE, genetic variation in plasticity) (Koch & Guillaume, 2020; Levine et al., 2011; Rockman, 2008; Zhou et al., 2012) (Figure 1). Integrating measurements of gene expression and physiology for organisms exposed to different environments allows us to clarify how much of the organism's response is determined by the environment (E) versus the variation between genotypes in their response to the environment (GxE).

This study focuses on responses to environmental change in the eastern oyster, *Crassostrea virginica*, along the northern Gulf of Mexico (nGOM). Previous transcriptomic studies have found that *C. virginica* alters its gene expression in response to environmental variation and disease state (Jones et al., 2019; Proestou & Sullivan, 2020). In the nGOM, salinity is one of the main environmental variables affecting the distribution and performance of this species (La Peyre et al., 2009; Parker, 1960; Rybovich et al., 2016). Although oysters inhabit a broad range of salinities, these conditions are also expected to change rapidly in the coming decades. Populations are currently exposed to salinities ranging from 4 to 35 psu, and local salinity regimes are expected to change rapidly due to anthropogenic alterations to coastal hydrology and intensification of storm events, along with changes in rainfall patterns (Bishop et al., 2019; Cayan et al., 2010; Das et al., 2012).

Salinity also interacts with biotic pressures in this region, with negative effects of salinity being greater at the lowest salinities and biotic pressures being greatest at higher salinities (Chu et al., 1993; Leonhardt et al., 2017). *Perkinsus marinus* is the cause of a lethal disease, commonly known as dermo, in the eastern oyster and is most prevalent at salinities greater than 10–12 psu (Andrews, 1988; Chu & Greene, 1989; Ragone Calvo et al., 2003). Previous research has shown *C. virginica* alters the expression of genes related to immune function in response to being challenged with *P. marinus* (Proestou & Sullivan, 2020; Tanguy et al., 2004; Wang et al., 2010). However, there remains a major gap in our understanding of how genotype, environment and GxE interaction influences the immune response to this parasite.

To investigate the roles of plastic and evolved responses to environmental change and *P. marinus* infection in *C. virginica*, we employed a space for time substitution (Pickett, 1989) and used a common garden experiment with 80 oysters from six families outplanted at two field sites naturally differing in salinity regimes. Our objective was to determine the relative contribution of plasticity, evolution and the evolution of plasticity in *C. virginica's* response to changes in salinity and *P. marinus* infection intensity. To accomplish this, we combined measurements of growth, *P. marinus* infection and 3′ RNA sequencing (TagSeq) to identify the effect of genotype, environment and genotype-by-environment interaction on the oyster's response to environmental variation. Additionally, we assessed the sequence evolution for genes showing plastic or evolved responses using genomic data from an ongoing low-coverage whole genome project comparing 20 oysters from nine populations in the nGOM (K. A. Sirovy et al., 2020). As one of the first studies to characterize the joint effects of genotype and environment on gene expression profiles in a natural setting, our study provides insights into the highly plastic and genetically limited response of *C. virginica* to environmental variation.

2 | MATERIAL AND METHODS

2.1 | Oyster conditioning and outplanting

In May 2016, we collected adult *C. virginica* oysters by dredging at three Louisiana, USA, estuaries: Vermilion Bay (29°36′39.99″N, 92°3′19.70″W), Sister Lake (29°12′50.70″N, 90°56′3.12″W), and Calcasieu Lake (29°51′00″N, 93°18′40″W) (Table S1). The adults were transported to the Louisiana Department of Wildlife and Fisheries Michael C. Voisin (MCV) Oyster Hatchery in Grand Isle, LA (29°14′20.3″N, 90°00′11.2″W), and placed into off-bottom mesh cages for acclimation. In October 2016, after 6 months of acclimation, the oysters were spawned at the MCV oyster hatchery. Unintentionally, only one population was used for the crosses and the specific population used was not recorded, but oysters from

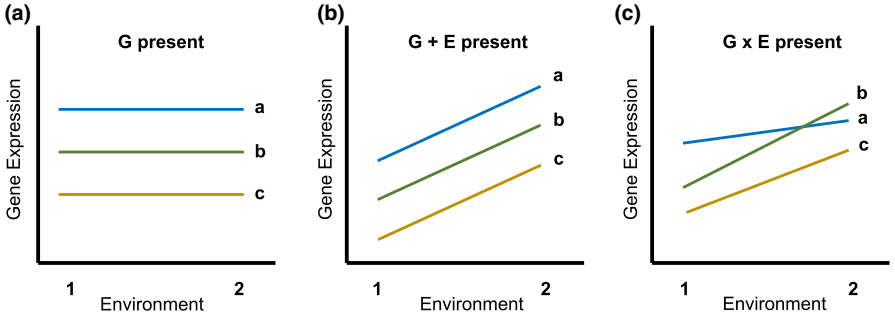

FIGURE 1 Hypothetical reaction norms for three genotypes showing an effect of (a) genotype, (b) genotype and environment and (c) genotype-by-environment interaction [Colour figure can be viewed at wileyonlinelibrary.com]

all three of these collection sites are genetically similar (Johnson & Kelly, 2020). The hatchery performed a 3 male × 2 female cross from this single population, resulting in six full-sib families.

After the oyster larvae were set, the spat stage was reared in an upwelling system until reaching >15 mm mean shell height, at which point the oysters were individually tagged and outplanted in one of three adjustable long-line mesh bags at both the Grand Isle Hatchery farm and near the Louisiana Universities Marine Consortium (LUMCON) (29°15′12.6″N, 90°39′45.9″W) on February 20, 2017. The hatchery did not sort oysters based on family of origin, resulting in an uneven number of individuals from each family being outplanted (see Supplemental Material: Oyster_data.csv). The outplant sites naturally differ in salinity regimes, with LUMCON representing a low-salinity site and Grand Isle representing a medium- to high-salinity site. Oysters were assessed for mortality and cleaned of epibionts approximately every 3 months over a 14-month period. Temperature and salinity conditions were measured by the closest USGS monitoring station (USGS 073802516 Barataria Pass at Grand Isle, LA) or by the consortium itself for LUMCON (Figure S1). The mean (±*SD*) temperature and salinity during the 14-month outplant was 23.0 (±6.2)°C and 11.2 (±5.6) psu at LUMCON and 22.4 (±5.9)°C and 20.7 (±6.5) psu at Grand Isle.

2.2 | Sample collection

On April 24, 2018, at the end of the 14-month outplant, 40 of the tagged individuals were sampled at each site. Shell height was measured from shell umbo to distal edge using a digital caliper (Mityoto USA). For gene expression analysis, an ~0.5-cm^2 piece of gill tissue was dissected in the field from each individual and preserved with Invitrogen RNA*later*. The remaining tissue was blotted and placed in a preweighed 50-ml test tube to measure wet meat weight. *Perkinsus marinus* infection intensities were evaluated by adding 0.22 μm filtered seawater (20 psu) at a concentration of ~0.4 g ml^{-1} and homogenizing the oyster tissue in each 50-ml test tube. One millilitre of the oyster homogenate was used to measure the number of *P. marinus* parasites per gram of oyster wet tissue using the whole-oyster procedure (La Peyre et al., 2018).

2.3 | Morphometrics and infection intensity

We compared final height, wet meat weight and infection intensities between sites and families. We used the Shapiro–Wilk normality test (Royston, 1982; Shapiro & Wilk, 1965) to determine if the height, weight and infection intensity data followed a normal distribution. Oyster height and weight data were normally distributed after square-root transformation (Shapiro-Wilk test $p > .05$). The height and weight data were fitted with a linear mixed-effect model with bag as a random factor using the R package LME4 (Bates et al., 2015). A two-way ANOVA was performed using the CAR package for both height and weight ($p < .05$) (Fox & Weisberg, 2011). The infection intensity data were not normally distributed and were therefore analysed using the nonparametric Kruskal–Wallis Rank Sum test ($p < .05$) (Hollander et al., 2013; Kruskal & Wallis, 1952). *Perkinsus marinus* infection intensities differed greatly by outplant site (Figure 2), so to reduce environmental noise we also analysed infection intensity at each site separately using the Kruskal–Wallis test ($p < .05$). To examine pairwise multiple comparisons we used the post hoc Dunn's test with the Benjamini–Hochberg correction ($p < .05$) (Benjamini & Hochberg, 1995).

2.4 | Gene expression analysis; sampling and initial processing

Total RNA was extracted using an E.Z.N.A. Total RNA Kit I (Omega BIO-TEK; VWR catalogue no. 101319) following the manufacturer's instructions. The yield and quantity were initially assessed using a NanoDrop 2000 spectrophotometer. Total RNA extracted from the 80 individuals was sent to the University of Texas at Austin's Genomic Sequencing and Analysis Facility where RNA quality control was confirmed using a 2100 Agilent Bioanalyzer on a Eukaryote Total RNA Nano chip and libraries were produced using the Tag-sequencing approach (Meyer et al., 2011). The resulting 80 libraries were sequenced equally across two lanes of an Illumina HiSeq 2500 platform, with 100-bp single-end reads.

Sequencing reads were trimmed of adapter sequences using TRIMMOMATIC (version 0.39) (Bolger et al., 2014) and base pairs with quality scores below 30 were removed (Table S2). The trimmed reads were mapped to the *C. virginica* reference genome (Gómez-Chiarri et al., 2015) with known haplotigs removed (https://github.com/jpuritz/OysterGenomeProject/tree/master/Haplotig_Masked_Genome) using the single pass option for STAR RNA-SEQ ALIGNER (version 2.6.0a) (Dobin et al., 2013). Reads were mapped to gene features with the options (--quantMode GeneCounts --outFilterScoreMinOverLread 0.50 --outFilterMatchNminOverLread 0.50) specified to adjust for poly-A tail contamination. A count matrix was generated from the ReadsPerGene.out.tab output from STAR (Data S1). Genotypes for each individual were called using ANGSD (version 0.931) to produce an identity-by-state matrix. The filters used for assigning identity-by-state scores included removing sites with allele frequency lower than 0.05, requiring a minimum read mapping quality score of 30, a minimum base mapping quality above 20, and removing single nucleotide polymorphisms (SNPs) with a *p*-value >2e−6. These filters allow for high stringency and align with previously published work that has used ANGSD to assess genotypes in wild populations (Sturm et al., 2020). Genotype clusters were identified by plotting the first two axes from a distance-based redundancy analysis with the cap-scale function in the R program VEGAN (version 2.5-6).

2.5 | Gene expression analysis; EDGER and PCoA

In light of the growing evidence that negative-binomial distributions perform poorly on published RNA-seq data (Assefa et al., 2018;

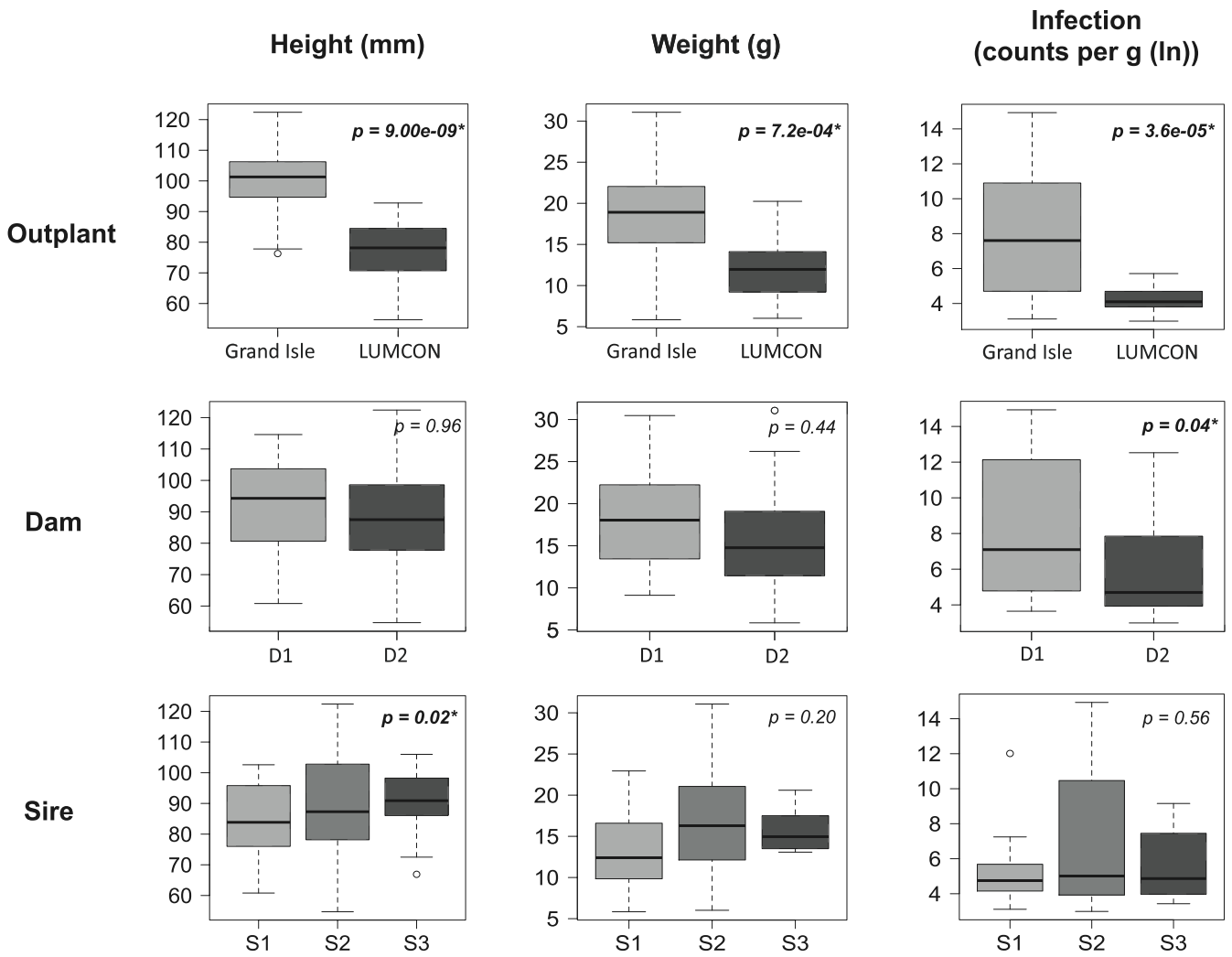

FIGURE 2 Measuring the effect of outplant, dam and sire on final shell height (mm), wet meat weight (g) and *Perkinsus marinus* infection intensity (counts per gram [ln]). Significant effects are bolded with an asterisk ($p < .05$)

Benidt & Nettleton, 2015; Hawinkel et al., 2020), we decided to use three distinct statistical approaches to ensure robustness. The first approach incorporates traditional assumptions (negative-binomial distribution) to assess pairwise changes in gene expression associated with genotype (sire and dam) and environment (outplant site) using the package EDGER (version 3.24.2) (Robinson et al., 2010). We used the command (filterByExpr) to filter our genes and the remaining read counts were normalized using a trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack, 2010). Broad changes in gene expression were first assessed using a principal coordinate analysis (PCoA) conducted using the R program VEGAN with euclidean distances calculated from $\log_2$ +1-transformed normalized counts obtained from the cpm() function in EDGER. Pairwise differential expression was measured using the genewise negative binomial generalized linear model implemented in the EDGER function glmQLFit and significantly differentially expressed genes (DEGs) were identified based on FDR rates calculated using Benjamini–Hochberg method (Benjamini & Hochberg, 1995). Functional enrichment of DEGs was tested using a Fisher's Exact Test ($p < .05$). All Fisher's Exact Tests were conducted using the scripts originally developed by Wright et al. (2015) and available at: https://github.com/z0on/GO_MWU/blob/master/GO_MWU.R. This method uses a binary input (DEGs =1, non-DEGs =0) to calculate if there is enrichment in the DEGs across three gene ontology (GO) categories: Molecular Function (MF), Biological Processes (BP) and Cellular Component (CC).

2.6 | Gene expression analysis; PERMANOVA

The second approach used a PERMANOVA to identify genes associated with genotype, environment or GxE interaction on both total gene expression as well as on a per-gene basis. The PERMANOVA is a nonparametric test and thus offers a complementary method to offset the negative-binomial assumptions from EDGER (Anderson, 2014). For both PERMANOVAs, log-transformed counts were evaluated using the R function 'adonis2' within the VEGAN package (version 2.5-6). The PERMANOVA examined the

effect of gene expression ~ sire + dam + outplant + bag + sire * outplant + dam * outplant with 10^6 permutations (total expression) or 9999 permutations (per-gene expression) ($p < .05$). The difference in the number of permutations between the total expression and per-gene analysis is the result of the higher computational requirements for the per-gene analysis. For the per-gene PERMANOVA, the *p*-values were corrected for multiple comparisons using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995). To reduce environmental noise, we also ran a PERMANOVA for each site separately to identify genes associated with shell height and infection. This was done because the two sites differ greatly in infection intensities and salinity regime. Functional enrichment of significant genes was tested using a Fisher's Exact Test ($p < .05$).

2.7 | Gene expression analysis; WGCNA

The third approach used to assess changes in gene expression was a weighted gene co-expression network analysis (WGCNA). In contrast to the previous two approaches, a WGCNA uses correlation networks to find clusters of genes with highly correlated expression patterns (Langfelder & Horvath, 2008). For all WGCNAs we restricted the number of genes to remove lowly expressed features retaining only samples with greater than five counts per million in 80% of all samples. WGCNA was run using the 11,214 genes that passed this filter, a soft-threshold of 16, a minimum module size of 30 and a signed adjacency matrix, and was correlated to sire (a binary variable for sire 1, 2 and 3), dam (a binary variable for dam 1 and 2) and outplant (a binary variable for Grand Isle or LUMCON). To reduce environmental noise, we also applied a WGCNA for height and infection at each site separately. Functional enrichment of significant modules was tested for using a Fisher's Exact Test ($p < .05$).

2.8 | Characterizing DEGs and WGCNA modules; expression levels, Ka/Ks ratios and nucleotide diversity (Pi)

To test whether DEGs or genes from significant WGCNA modules showed distinctive patterns in terms of expression levels, we used the average log-transformed counts (cpm) from EDGER as our count matrix. Genes identified from EDGER, PERMANOVA or WGCNA were analysed separately. We compared the expression levels of genes correlated to genotype, environment or infection intensity to the average expression level for all genes using the bootstrap version of the Kolmogorov–Smirnov (KS) test ($p < .05$) (R function 'ks.boot' from package MATCHING version 4.9-7).

We estimated the nonsynonymous to synonymous substitution (Ka/Ks) ratios for each gene using data from an ongoing project where we have collected low-coverage whole genome data for 20 oysters from each of nine populations ($n = 180$) in the nGOM spanning from Port Isabel, Texas, to Lake Fortuna, Louisiana (K. A. Sirovy et al., in preparation) (Table S3). DNA libraries were sequenced on an Illumina HiSeqX platform, with 150-bp paired-end reads. Sequencing reads were trimmed of adapter sequences using TRIMMOMATIC (version 0.39) (Bolger et al., 2014) and base pairs with quality scores below 30 were removed. The trimmed reads were mapped to the *C. virginica* reference genome (Gómez-Chiarri et al., 2015) using BWA (Burrow's Wheeler Aligner) (Li & Durbin, 2009) followed by removal of duplicates and indexing using PICARD TOOLS software (https://github.com/broadinstitute/picard). We estimated the number of SNPs using the GATK pipeline (version 4.0.2.1) and annotated those SNPs using SNPEFF (Cingolani et al., 2012). Ka/Ks ratios were then calculated based on the VCF outputs from SNPEFF using a script available online at https://github.com/MerrimanLab/selectionTools/blob/master/extrascripts/kaks.py. Ka/Ks scores were filtered to keep values above 0 and below 3 to limit bias from outliers and significant contrasts were tested down to a ratio of 1.

We generated the nucleotide diversity (Pi) for the coding + intron, upstream and downstream region using reduced representation bisulphite sequencing data for the same 80 oysters (K. M. Johnson et al., 2020). SNPs were called using the program BS-SNPER while requiring a minimum heterozygous SNP frequency of 0.1; a minimum homozygous SNP frequency of 0.85; --minimum base quality of 15; a minimum depth of covered reads of 10; a maximum depth of covered reads of 1,000; a minimum mutation reads of 2; a minimum mutation rate of 0.02; and a Minimum read mapping value of 20 (Gao et al., 2015). These settings follow default conditions and are established to provide high quality SNPs from reduced representation bisulphite-converted sequence data. The VCFs were normalized, indexed and converted to BCFs using BCFTOOLS (version-1.10.2). Nucleotide diversity was then calculated using the program VCFTOOLS (version-0.1.14) with 1000-bp windows and a step size of 1000 bp.

To assess the nucleotide diversity and Ka/Ks levels for DEGs identified by EDGER, PERMANOVA or for significant WGCNA modules, we used a series of permutation tests to compare the mean nucleotide diversity (Pi) or Ka/Ks ratio of the genes and modules correlated to genotype, environment or infection intensity against the genome-wide average. Then, using the same number of significant genes or genes within a WGCNA module, the nucleotide diversity and Ka/Ks values were shuffled in R using the sample function to generate a new difference in means and this was repeated 10,000 times to generate a null distribution. This allowed us to test if the mean difference for the significant genes or modules was significantly different from the mean difference in the permuted list ($p < .05$). 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.

Nucleotide diversity and Ka/Ks ratios were also compared to average expression levels which had been binned using the R function 'decile' within the package STATMEASURES (version 1.0). A Kruskal–Wallis test was performed comparing the nucleotide diversity or Ka/Ks values with the 10 gene expression bins, followed by a Dunn's test with the Benjamini–Hochberg correction ($p < .05$) (Benjamini & Hochberg, 1995).

3 | RESULTS

3.1 | Morphometrics and infection intensity

To better understand the role of environment and genotype on oyster performance, we measured the final height (n = 80), wet meat weight (n = 66) and *P. marinus* infection intensity (n = 61) for outplanted oysters (Figure 2). Overall, environment had the strongest effect on shell height, wet meat weight and *P. marinus* infection intensity when both sites were included in the linear fixed-effect model, although a bag effect for infection intensity was also observed (Figure 2; Table S4). Dam showed a smaller but still significant effect on infection intensities when both sites were included (Figure 2; Table S4). Sire showed a significant effect on height in the model with both sites (Figure 2; Table S4) along with a significant effect on infection intensity when testing Grand Isle independently (Table S5). Dunn's test did not find any particular sire driving these patterns (p > .05). There were no effects detected in the analysis looking at LUMCON separately, although there was a nearly significant effect of sire on infection intensity (p = .07) (Table S6). We did not observe any interactive effects of outplant-by-sire or outplant-by-dam on oyster size or infection intensity. Infection intensities were both higher and more variable at the Grand Isle site (Figure 2).

3.2 | Summary statistics of TagSeq

Transcriptome sequencing using TagSeq produced a total of 408 million reads, with 5.1 million reads per sample (range: 3,273,930–8,756,037). Trimming led to a final read count of 4.92 million per sample, which is sufficient for the TagSeq method (Meyer et al., 2011). Star mapping resulted in 91.00% of reads mapping to the reference genome (uniquely mapped reads and multimapped reads averaged 66.83% and 24.17%, respectively). Filtering mapped reads based on expression resulted in a total of 21,388 genes kept for all downstream analyses.

3.3 | Gene expression analyses; PCoA, EDGER and PERMANOVA

PCoA revealed a large effect of outplant site on gene expression and a smaller but still significant effect of dam and sire (Figure 3). The PERMANOVA incorporating total gene expression identified a significant effect of outplant (p = 1e−06), sire (p = 4e−06) and dam (p = 7e−05), with no effect of bag (p = .14), and no interactive effects on gene expression (Table S4).

Genes responding to environment were identified using both EDGER and a PERMANOVA testing on a per-gene basis. For EDGER, we compared the gene expression profiles of individuals from LUMCON versus Grand Isle and detected 4525 genes (1871 up-regulated and 2654 down-regulated) responding to environment. In contrast, the PERMANOVA identified 7454 genes associated with environment, with 4222 of those genes overlapping with the EDGER results (Figure S2). GO enrichment detected five (MF) and 13 (BP) categories shared between the two analyses including *translation initiation factor activity*, *carbohydrate biosynthetic process* and *cofactor binding* (Table 1; Table S7).

Next, we tested for genes associated with genotype. For EDGER, we compared the expression between dams or sires regardless of outplant site. This resulted in 3410 genes responding to genotype, 1201 associated with dam and 2955 associated with sire. The PERMANOVA identified 4566 genes responding to genotype, with 2532 of those genes overlapping with EDGER (Figure S2). Functional enrichment only detected a single gene ontology, *zinc ion binding*, which came from the PERMANOVA results.

There were 858 and 1426 genes that overlapped between environment and genotype for EDGER and PERMANOVA, respectively; 512 of these genes overlapped between the two analyses. Only one GO enrichment, *glutamate biosynthetic process*, was detected for the PERMANOVA results. Although many genes overlapped between environment and genotype (G + E), we did not detect any genes responding to genotype-by-environment interaction (GxE). We also used the PERMANOVA to test for a correlation between gene expression and height or infection at either outplant site, but we did not detect any genes correlated with either of these two variables.

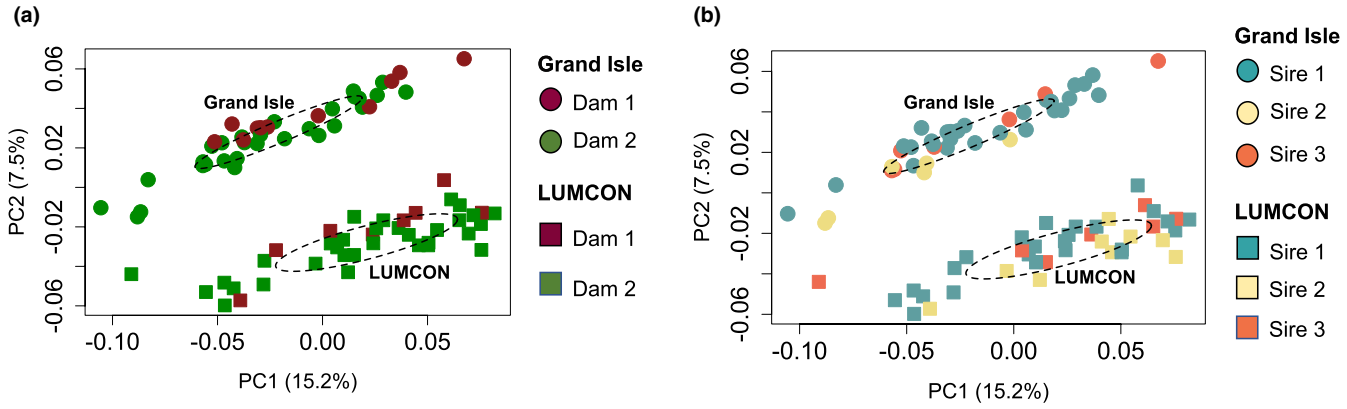

FIGURE 3 Principal coordinate analysis (PCoA) plot showing distances between samples. The PCoA was performed on gene counts that had been filtered and normalized using EDGER. The shapes represent the two outplant sites and colours differentiate parents: (a) dams and (b) sires [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Top enriched GO terms identified by all three analyses (EDGER, PERMANOVA and WGCNA) for genes responding to environment

GO category	GO term	Data set	Analysis	Response	GO ID
MF	Translation initiation factor activity	Full	All three	Environment	GO:0003743
MF	Transferase activity	Full	All three	Environment	GO:0008168; GO:0016741
MF	Cofactor binding	Full	All three	Environment	GO:0048037
BP	Carbohydrate biosynthetic process	Full	All three	Environment	GO:0006094; GO:0006006
BP	Nucleic acid-templated transcription	Full	All three	Environment	GO:0006351; GO:0097659
BP	RNA processing	Full	All three	Environment	GO:0006396
BP	RNA metabolic process	Full	All three	Environment	GO:0016070
BP	Monosaccharide metabolic process	Full	All three	Environment	GO:0019318; GO:0005996
MF	Structural constituent of ribosome	LUMCON Only	WGCNA	Infection	GO:0003735
BP	Cellular amide metabolic process	LUMCON Only	WGCNA	Infection	GO:0043603; GO:0006518
CC	Ribonucleoprotein complex	LUMCON Only	WGCNA	Infection	GO:1990904

Note: GO terms identified by WGCNA for infection are also listed.

3.4 | WGCNA

The WGCNA was used to detect groups of genes responding similarly to environment, genotype, height or infection. A gene co-expression matrix was generated with modules that represent similarly expressed genes. We found that when examining both sites ($n = 80$), 10 out of 14 total modules were significantly correlated with outplant (Figure S3). However, there were no modules associated with dam or sire. Functional enrichment revealed 24 (MF), 44 (BP) and 22 (CC) gene ontologies associated with environment, including 17 ontologies that overlapped with EDGER and PERMANOVA. The overlapping ontologies consisted of *nucleic acid-templated transcription*, *RNA processing* and *monosaccharide metabolic process* (Table 1; Table S7).

To reduce environmental noise, we also applied a WGCNA for genotype ($n = 40$), height ($n = 40$) and infection ($n = 24$ for LUMCON and $n = 39$ for GI), analysing each site separately (Figure S3). For Grand Isle, we detected five modules associated with genotype and no modules correlated to height or infection. For LUMCON, there were two modules correlated with infection, but no modules associated with height or genotype. GO enrichment did not detect any categories associated with genotype at Grand Isle. Enrichment for genes correlated to infection at LUMCON revealed three (MF), six (BP) and eight (CC) gene ontologies comprising *structural constituent of ribosome*, *ribonucleoprotein complex biogenesis* and *cellular amide metabolic process* (Table 1; Table S7).

3.5 | Characterizing differentially expressed genes; expression levels

Genes responding to genotype from all three analyses had lower mean expression levels compared to background levels (Figure 4a), with one exception for the "red" module from WGCNA which showed elevated levels associated with sire 3 (Table S8). In contrast, genes associated with environment had elevated expression levels (Figure 4B). Genes that were shared between environment and genotype were also elevated relative to background expression levels (Figure S4). The two modules correlated with infection rates at LUMCON also showed increased expression levels (Table S8).

3.6 | Characterizing differentially expressed genes; Ka/Ks ratios

We calculated Ka/Ks values for 10,914 genes after filtering. Genes associated with genotype from all three analyses had elevated Ka/Ks ratios compared to background levels (Figure 4c; Table S8). In contrast, genes responding to environment showed lower Ka/Ks values with the exception of a WGCNA module "midnightblue" which had elevated ratios (Figure 4d; Table S8). For genes that were differentially expressed due to both environment and genotype, Ka/Ks was lower, but this was only significant based on the PERMANOVA results (Figure S4). The WGCNA "tan" module correlated with infection at LUMCON showed increased Ka/Ks values (Table S8). Finally, we compared Ka/Ks ratios against expression levels and found a negative correlation between Ka/Ks and gene expression (Figure 5a).

3.7 | Characterizing differentially expressed genes; nucleotide diversity (Pi)

Nucleotide diversity was calculated for coding + intron (16,520 genes), upstream (5766 genes) and downstream (6044 genes)

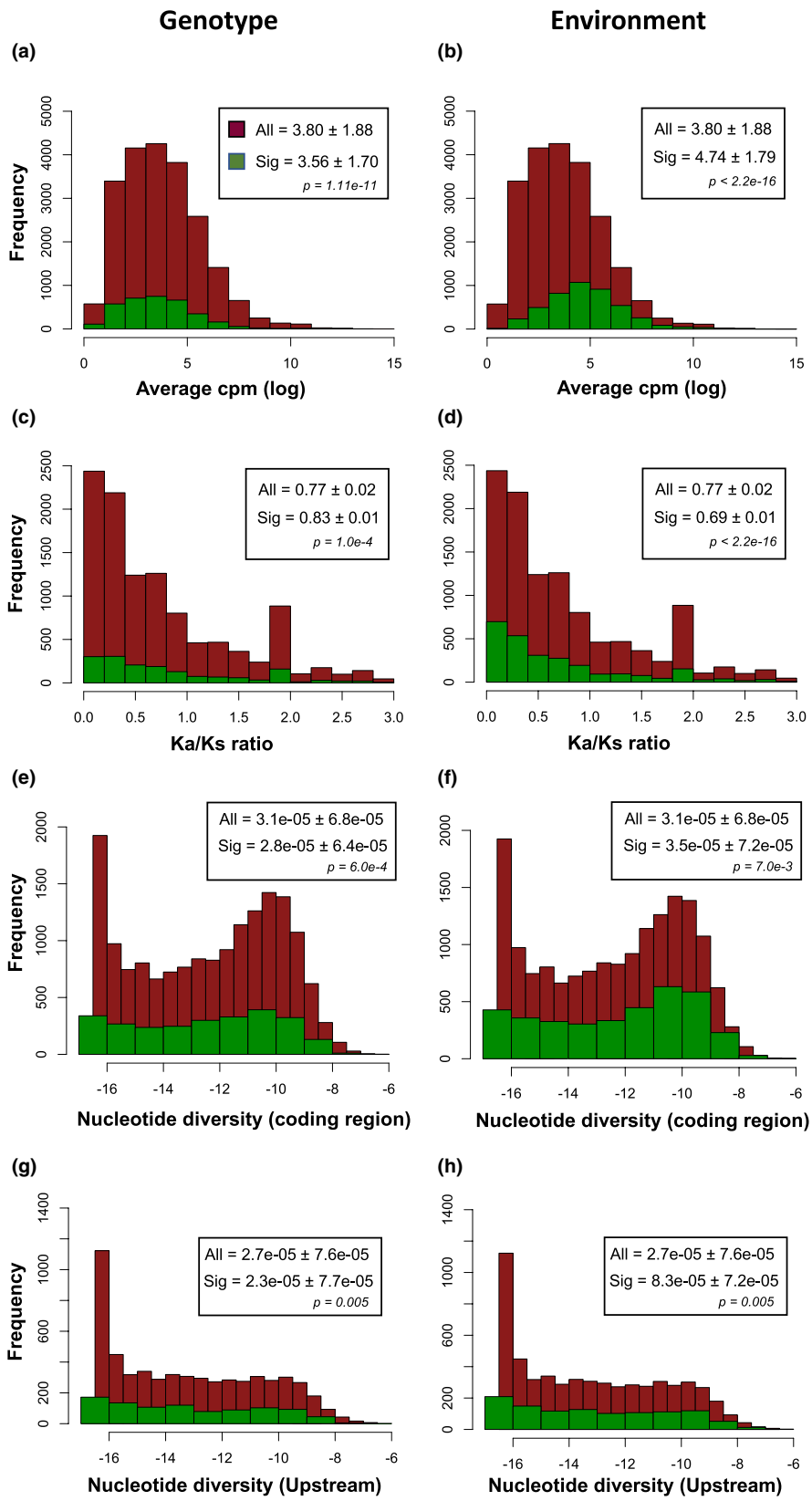

FIGURE 4 Histograms showing the frequency of genotype and environment genes at different levels of (a, b) gene expression, (c, d) Ka/Ks ratios, (e, f) nucleotide diversity (Pi) in coding regions + introns, and (g, h) nucleotide diversity (Pi) in upstream regions. Because all three analyses (EDGER, PERMANOVA, WGCNA) show nearly identical results, we present only the PERMANOVA values to simplify the findings [Colour figure can be viewed at wileyonlinelibrary.com]

regions. Genes associated with genotype for all three analyses had decreased nucleotide diversity in the upstream and coding region compared to background levels (Figure 4e,g; Table S8). In contrast, genes responding to environment had increased nucleotide diversity among all regions (Figure 4f,h). Differences in the upstream region for both environment and genotype genes were only significant based on the PERMANOVA analysis, but the EDGER results shared a similar pattern but were not significantly different from background

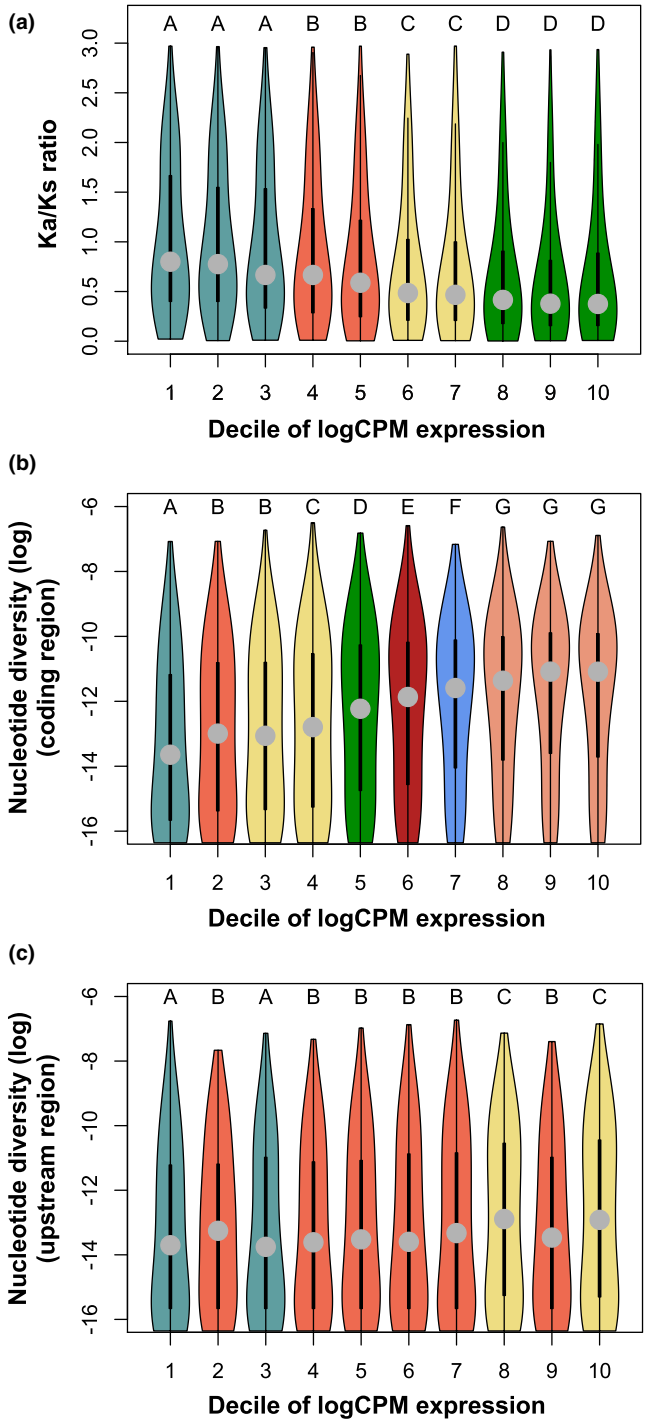

FIGURE 5 Violin plots showing average expression levels across all 80 oysters plotted against (a) Ka/Ks ratios, (b) nucleotide diversity (log) (Pi) of coding region + introns and (c) nucleotide diversity (log) (Pi) of the upstream region. Expression levels are plotted from lowest to highest [Colour figure can be viewed at wileyonlinelibrary.com]

levels. Genes that were shared between environment and genotype did not differ from background levels (Figure S4). Interestingly, the two WGCNA modules correlated with infection showed opposing patterns, with genes in the "pink" module having elevated diversity and the genes in the "tan" module having decreased diversity in both the coding and upstream region ($p < .05$) (Table S8). Finally, we compared nucleotide diversity against expression levels and found a positive correlation between diversity (Pi) and gene expression (Figure 5b,c).

4 | DISCUSSION

We present one of the first studies to combine transcriptomics and physiology to characterize the reaction norms of families reared in a common garden at two field sites in a wild animal. This experimental design allowed us to assess the influence of plastic and evolved responses on gene expression and physiology. By using a comprehensive approach with three unique transcriptomic analyses combined with growth data and *P. marinus* infection intensities, we were able to identify the effect of genotype (G), environment (E) and genotype-by-environment interaction (GxE) on the oyster's response to environmental variation. Our RNAseq analyses (EDGER, PERMANOVA and WGCNA) mainly agree in finding a larger number of genes responding to environment rather than genotype, as well as a lack of GxE interaction (Figure S2). Additionally, results of all three analyses are in agreement and indicate that genes differing by genotype have opposing characteristics in terms of expression levels, Ka/Ks ratios and nucleotide diversity as compared to genes responding to environment. Overall, we found that *C. virginica* has a highly plastic response to environment; however, that plasticity is largely uniform across genotypes (Figure 1b).

4.1 | Highly plastic, yet genotypically similar response for growth and infection

We demonstrate that *C. virginica* exhibits high plasticity for growth and *P. marinus* infection resistance in response to environment and that plasticity is largely parallel among genotypes (Figures 1b and 2). We did find a small effect of sire on final shell height and dam on infection intensity, but we did not detect any GxE interaction. The research focused on GxE in bivalves is divided, with some studies finding very little GxE in response to environmental change (Evans & Langdon, 2006; Kvingedal et al., 2008), while other studies find extensive GxE (Hughes et al., 2017; Proestou et al., 2016; Rawson & Hilbish, 1991). Our study suggests that *C. virginica's* response to environmental variation among sites falls into this first group of studies, lacking genetic variation in its plastic response to environmental change. However, it is possible that we did not have a broad enough range of genotypes to capture GxE or that differences in plastic responses between genotypes was a result of alternatively spliced isoforms which are not captured using the TagSeq method. Regardless, our ability to detect differences between genotypes suggests that if GxE is present it is smaller than G in this setting. With regard to infection intensity, the prevalence of *P. marinus* infection was positively correlated with salinity. The range of infection intensities was much larger at the higher salinity site, probably

allowing us to detect an effect of sire, although this could be the result of a bag effect (Figure 2). Together, our findings suggest that *C. virginica* may be able to buffer the immediate impacts of changing conditions through changes in gene expression, although they may be limited to the range achieved in the initial response, as the lack of genetic variation in plasticity suggests limited capacity for evolutionary change in this setting.

4.2 | Influence of genotype versus environment on gene expression

Of the 21,388 genes used in this study, 21%–35% (EDGER–PERMANOVA) were differentially expressed in a plastic manner while 16%–21% of the genome showed differences in mean expression among genotypes, regardless of environment. Genes responding to environment versus genotype had distinct profiles with regard to gene expression, Ka/Ks ratios and nucleotide diversity. Genes associated with genotype had low expression levels, high Ka/Ks ratios and low nucleotide diversity in both the coding and the upstream region. Environment genes displayed the exact opposite characteristics, showing high expression levels, low Ka/Ks ratios and high nucleotide diversity in coding and upstream regions.

Why would genes responding to environment versus genotype have opposing profiles? We found that gene expression levels are positively correlated with nucleotide diversity, but negatively correlated with Ka/Ks ratios (Figure 5). The negative correlation with Ka/Ks is expected since mutations in highly expressed genes are often subject to stronger purifying selection because these genes are more likely to have an important role in housekeeping functions (Carneiro et al., 2012) and are expected to be constrained by a higher sensitivity to changes in downstream protein folding (Drummond et al., 2005; Park et al., 2013). However, the positive correlation between expression levels and nucleotide diversity (Pi) in the face of strong purifying selection is surprising. One possible explanation is that gene expression itself has a mild mutagenic effect which could elevate nucleotide diversity (Lynch et al., 2016). Alternatively, methylation has been shown to be positively correlated with gene expression in this same set of oysters (Johnson et al., 2020) and this epigenetic signature is known to have large mutagenic effects (Gonzalgo & Jones, 1997; Pfeifer, 2006) which may also lead to an increase in nucleotide diversity. Overall, these findings suggest that genes responding to environment are more important to the performance of *C. virginica*, and genes associated with genotype are under a more relaxed selection process.

GO analysis gave us the ability to discover biologically relevant categories for genes associated with environment and genotype. *RNA metabolic process* and *organic cyclic compound biosynthetic process* were among the environmental GO terms we detected and both have been found in previous studies comparing *C. virginica* exposed to changing temperature and salinity (Jones et al., 2019). Additionally, there were several GO terms for environment that were associated with glutamate metabolisms which have been shown to help regulate osmotic stress through proline accumulation in copepods and Chinese mitten crabs (Wang et al., 2012; Willett & Burton, 2003). Even more interesting than any particular GO terms was that the DEGs associated with environment were enriched for over 100 categories while genes associated with genotype had just one significant grouping. This pattern is surprising as the number of DEGs for genotype and environment are relatively large and similar. The large number of DEGs associated with genotype indicates that *C. virginica* has a high capacity to evolve differences in mean expression of these genes. However, the lack of enriched GO terms for genotype suggests that selection has not targeted any specific biological category, but rather a broad array of functions. The large number of DEGs associated with environment suggests a high capacity for plasticity, and the 100+ enriched categories suggests that these functions are biologically relevant for the performance of this oyster across environments and are thus more likely to be involved with responses to future environmental change.

Lastly, we looked at genes responding to both genotype and environment, as these genes are potential drivers for the evolution of plasticity. GxE is present whenever the reaction norms of at least two genotypes are not parallel. We used a PERMANOVA test to examine reaction norms for gene expression and found that all genotypes had parallel transcriptomic responses across environments, indicating the absence of GxE. Our gene expression results, in agreement with our physiology data, suggest that *C. virginica* has a highly plastic response to environmental changes, but that response appears to be generalized across genotypes which may limit the organism's ability to evolve plasticity to future environmental stressors (Figure 1b).

4.3 | Gene expression; not a major driver of growth or infection intensity

Although we found that genotype influences growth and infection, this influence does not appear to be driven by gene expression. WGCNA detected two modules associated with infection at Grand Isle, suggesting that the expression patterns of genes within these modules are significantly correlated with both growth and infection. GO enrichment found that these genes are involved in processes regulating translation and peptide formation, although there were no categories that had an explicit connection with immune response. Since RNA sequencing only captures a snapshot in time, our failure to detect a large effect of gene expression on growth and infection may be due to sampling mRNA at a time that was not relevant to these traits. For example, in contrast to our findings, Proestou and Sullivan (2020) found distinct expression profiles for acute versus acclimatory responses to *P. marinus* infection, although this was mainly dependent on time after exposure. Proestou and Sullivan (2020) included three time points (36 hr, 7 days and 28 days after exposure), and found that the majority of differential expression took place on day 28. This highlights the potential for our study to have simply missed sampling at a time that was relevant for gene expression. One last caveat is that the genotype could also influence

size and infection via protein coding differences rather than through changes in expression. The lack of studies focused on protein coding differences in the eastern oyster represents a major gap for future investigation.

4.4 | Lack of genotype-by-environment interaction (GxE)

The absence of genetic variation in plasticity for gene expression, growth and infection is not surprising since GxE has commonly been found to vary across studies (Gibbons et al., 2017; Harder et al., 2020; Kirk et al., 2018). There are many hypotheses for why GxE may vary between systems, which are thoroughly detailed in the review by Saltz et al. (2018). One potential hypothesis to explain variation in GxE is that genotypic groups with higher genetic variance should show greater GxE than genotypes with lower genetic variance. This is because more genetic variation increases the chance of containing genotypes which have unusual reaction norms and thus more GxE. Our results do not support this hypothesis because although we were able to detect a large number of genes responding to genotype, this did not correspond to any GxE. Additionally, one can also argue that an increase in an environmental effect will also correspond to an increase in GxE (Saltz et al., 2018). The reasoning behind this is that the more plastic a genotype is, the more opportunity there is to generate an unusual reaction norm as compared to a genotype that changes minimally between environments. However, again our results do not support this hypothesis as we detected a large number of genes responding to environment, but not GxE.

One potential reason for the lack of GxE is that response to environmental heterogeneity is important, and so selection has removed genetic variation in the slopes of the reaction norms for genes responding to site. This is consistent with our finding that genes responding to environment have lower Ka/Ks (under stronger purifying selection) whereas the genes responding to genotype have higher Ka/Ks, suggesting more relaxed selection (Figure 4c,d). Alternatively, as the genotypes in this study were limited to six full-sib families from a single population of oysters, it is possible that the genotype range is too narrow for the detection of GxE. With that said, our ability to detect such a large number of genes responding to genotype and environment gives us more confidence in this negative result. Ultimately, we need more studies reporting GxE in order to start thoroughly addressing why there is variation in the evolution of plasticity across systems.

5 | CONCLUSIONS

Our study demonstrates that *C. virginica* exhibits a highly plastic, but genotypically parallel transcriptomic and physiological response to changes in outplant site. This suggests that *C. virginica* may be able to immediately buffer future environmental changes by altering gene expression and physiology, although this may limit the oyster's capacity to evolve plasticity. Since this study focused on a single population, future research is essential to confirm that this lack of GxE holds across multiple populations spanning several environmental gradients. Furthermore, we show that the combination of transcriptomics and physiology data with a natural common garden design can be beneficial for quantifying the relative contributions of plasticity and genetic variation. This study provides a framework for partitioning the effects of genotype, environment and genotype-by-environment interactions using transcriptomics. Future studies are needed to better understand the relative contributions of plasticity, evolution and evolution of plasticity on phenotypic change in response to changing environments.

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CONFLICT OF INTEREST

We declare no competing interests.

AUTHOR CONTRIBUTIONS

M.W.K. and J.F.L. conceived of and designed the outplant study. K.M.J., M.W.K., J.F.L., S.M.C. and K.A.S. sampled the animals for this study. S.M.C. and J.F.L. measured infection intensities. K.M.J. prepared RRBS libraries and performed differential methylation analysis. K.A.S. and K.M.J. analysed the transcriptomic data and wrote the manuscript with input from S.M.C., J.F.L. and M.W.K. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The count data, oyster traits, list of GO terms and scripts used for analyses can be found on Dryad at https://doi.org/10.5061/dryad.wpzgmsbmt. The raw sequencing data can be found at the NCBI SRA database under BioProject submission PRJNA724997.

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SUPPORTING INFORMATION

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

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