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Reef-Specific Patterns of Gene Expression Plasticity in Eastern Oysters (*Crassostrea virginica*)

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Abstract

Understanding the interaction between phenotypic plasticity and evolutionary processes is important for predicting a species' response to changing environment. Strong recurrent selection each generation may be an important process in highly fecund species with broad dispersal and extensive early mortality. We tested whether selection was associated with spatial divergence in gene expression plasticity for osmoregulation in the eastern oyster (*Crassostrea virginica*). We collected adult oysters from high and low salinity reefs within a single estuary and after 9 weeks of acclimation at 10 and 30 salinity, measured gene expression in 24 oysters using next-generation RNA sequencing technology. The oysters had significantly different expression (DE) in response to salinity treatments for 7936 (18.9%) transcripts overall, with planned contrasts showing 8× more DE in oysters from the high-salinity reef and 15× more DE between reefs when tested at 10 salinity. The reef-by-treatment interaction was also genomically pervasive (5858 DE transcripts, 13.9%). Inter-reef F_{ST} for transcript SNPs averaged 0.0025 with the top 1% between 0.29 and 0.73. Transcripts containing "outlier" SNPs were significantly enriched for osmoregulatory genes and showed patterns of variation consistent with selection on the low-salinity reef. Both phenotypic plasticity and recurrent selection seem to be important factors determining the realized niche of oysters within estuaries.

Subject areas: Molecular adaptation and selection; Population structure and phylogeography
Key words: linkage disequilibrium, nucleotide diversity, osmoregulation, phenotypic plasticity, RNA-seq, viability selection

Managing for population persistence and evolutionary potential requires understanding the mechanisms by which individuals and populations respond to environmental change. The potential responses are dispersal, phenotypic plasticity, or adaptation, and each involves species- and environment-specific constraints that are often poorly known. In addition, expected interactions between these 3 responses lead to a dynamic currently described as eco-evolutionary (Kinnison and Hairston 2007; Pelletier et al. 2009). The interaction between plasticity and evolution is of particular interest because it may either augment or constrain local adaptation depending on the context (Ghalambor et al. 2007; Crispo 2008; Morris et al. 2014). In light of rapid anthropogenic environmental change, understanding the interaction between natural selection and physiological plasticity may be a useful first step for investigating how environmental and demographic contexts affect the evolution of highly plastic traits (Hendry et al. 2008; Hofmann and Todgham 2010; Nicotra et al. 2010; Evans and Hofmann 2012).

Phenotypic plasticity encompasses a broad range of morphological, physiological, and behavioral responses to environmental

variation. While this complex trait is the subject of much published theory about its evolution and adaptive significance (e.g., Bradshaw 1965; Via and Lande 1985; Sultan 1987; Via 1987; Scheiner 1993; DeWitt et al. 1998; Schlichting and Pigliucci 1998; Pigliucci et al. 2006; Ghalambor et al. 2007), only a rudimentary understanding of its molecular basis and heritability exist outside of a few traits in a few organisms (e.g., Colinet and Hoffmann 2012; Kelly et al. 2012; Bhatia et al. 2014). Phenotypic plasticity is often categorized as either developmental or labile. Developmental plasticity occurs during the development phase of the life cycle and once the phenotype of the individual is determined, it cannot be changed. In contrast, labile plasticity occurs throughout the life of an individual in response to environmental change and the resulting phenotype is reversible. Labile plasticity, including physiological traits, has received much less theoretical (but see Lande 2014) and empirical attention (reviewed in Brommer 2013), yet is an important mechanism determining organismal tolerances and differential fitness.

The mechanisms by which populations respond to environmental variation are influenced by both phenotypic plasticity and heritable genetic variation. Often, the relative spatial and temporal scale of environmental heterogeneity determines the relative importance of plasticity or adaptive genetic differentiation (Bradshaw 1965; Levins 1968; Scheiner 1993, 2013; Baythavong 2011; Gomez-Mestre and Jovani 2013). Temporal variability in the environment should favor labile plasticity so that physiological traits track the fluctuating environment (Lande 2014) and maintain homeostasis at higher levels of organization (Scheiner 1993). However, plastic traits are determined not just by the environment but also by the underlying genotypes (G × E), leading to variation among individual plastic responses (Scheiner 1993; Falconer and Mackay 1996). The variation among individuals' realized trait values provides the phenotypic variation necessary for natural selection (Pigliucci 2005). For organisms with high gene flow, dispersing as seeds or larvae, and with high early mortality, strong selection from spatially varying environmental conditions may shape location-specific reaction norms of plasticity (e.g., Côté et al. 2014).

Several aspects of the eastern oyster (*Crassostrea virginica*) make it a particularly interesting subject for testing the degree to which a physiologically plastic trait experiences spatially adaptive divergence at scales smaller than average dispersal distances. As sessile adults living in an estuarine habitat, oysters are exposed to a temporally variable environment where conditions change with every tide. As predicted by such environmental variation, the eastern oyster displays a high degree of phenotypic plasticity in traits ranging from morphology (e.g., Robinson et al. 2014) to physiology (Shumway 1996). Specifically, oysters must maintain cell volume homeostasis despite having hemolymph that is isotonic with seawater. Like other bivalves, oysters regulate cell volume by moving inorganic solutes and organic osmolytes into and out of their cells (reviewed in Evans 2009). Buffering of cell volume across varying osmotic conditions is an exquisite adaptation that emerges from the orchestrated plasticity of gene expression. By measuring the differential expression of genes in response to environmental variation, in this case salinity, candidate physiological functions can be inferred for genes (Gracey 2007). For example, previous work on the eastern oyster measured significant expression changes in 250 transcripts in response to salinity (Chapman et al. 2011). More recently, the genome and transcriptome of the Pacific oyster (*Crassostrea gigas*) have provided a valuable resource for identifying candidate genes (Zhang et al. 2012) with follow-up gene expression work identifying metabolic pathways used in osmoregulation (Meng et al. 2013).

Next-generation RNA sequencing technology (RNA-seq) allows for the quantification of gene expression and coding DNA sequence variation across thousands of genes without previous genomic information, a valuable aspect for studying species that lack a reference genome such as the eastern oyster. Individuals acclimatized to different environments are expected to show differences in gene expression as a result. Common-garden study designs acclimate individuals from distinct environments to a shared environment to test for differential expression attributable to source population differences (genetic or epigenetic). Comparisons across several common garden treatments allows for the measurement of differential gene expression in response to environmental variation (physiological plasticity) and tests for population source-by-environment (G × E) interactions. RNA-seq has the additional benefit of providing DNA sequence information for single nucleotide polymorphism (SNP) identification. After accounting for possibly biases inherent in scoring SNPs from transcripts, these data provide gene-specific information on genetic differentiation among populations.

In this study, our goal was to measure the relative importance of plasticity and evolutionary differentiation as mechanisms contributing to gene expression variation across salinity treatments and between oyster reefs. To accomplish this we conditioned adult Delaware Bay *C. virginica* from a down-bay, high salinity oyster reef and an up-bay, low salinity oyster reef for 9–10 weeks in high and low salinity common-garden tanks at a research hatchery. The source reef locations represent close to ideal (25–30 salinity) and marginal conditions (10) for eastern oysters (Swannack et al. 2014). Using RNA-seq to measure adult gene expression across 42 072 transcriptome sequences, we found thousands of transcripts with a plastic response to the treatments and thousands more with a significant reef source-by-treatment effect. Our experimental design does not completely control for epigenetic causes of reef differentiation so we also tested for evidence of disruptive selection in the coding sequences. Indeed, among high-F_{ST} outlier loci we found significant enrichment for osmoregulatory genes and population genetic patterns suggesting strong selection on the low-salinity reef oysters.

Materials and Methods

Sample Collection

Two hundred adult oysters were collected from each of 2 sites in Delaware Bay, United States, on 18 April 2011 (see Supplementary Figure S1 online). Oysters were collected by hand in the lower intertidal at the "high" site [salinity 20–25 based on "Cape Shore" site in Narváez et al. (2012); 39°04.10′N, 74°54.77′W] and by dredge at ~5 m depth at the "low" site [salinity 6.5–14.5 as reported for Arnolds Reef in Bushek et al. (2012); 39°23.055′N, 75°27.002′W]. Based on hourly salinity data from up-bay and down-bay dataloggers over the course of a year, the low salinity site experienced slightly greater salinity variation per month but a similar 19 unit range of total salinity variation over the year (see Supplementary Information and Supplementary Figures S1 and S2 online).

Oysters were conditioned in tanks at the Haskin Shellfish Research Laboratory of Rutgers University (details in Eierman 2014). Each 500 L tank contained UV-irradiated 1 mm filtered seawater either maintained at salinity 30 (high treatment) or diluted with distilled freshwater to salinity 10 (low treatment), to provide treatments approximately matching average "home" conditions

for each source population. The 4 experimental groups were high salinity reef source oysters acclimated to high salinity (HH) or low salinity (HL), and low salinity reef source oysters acclimated to high salinity (LH) or low salinity (LL). The HH and LL groups were considered "home" treatment groups with salinity acclimation treatments similar to average wild conditions, whereas the HL and LH were "away" treatment groups with salinities outside the range typically experienced in the wild based on biophysical models and reported salinity ranges (Gay and O'Donnell 2009; Bushek et al. 2012; Narváez et al. 2012).

After 9 weeks of acclimatization, each adult oyster was shucked at room temperature and a piece of gill tissue was immediately placed in RNALater® (Ambion) and stored at 4 °C until archiving at −80 °C within 2 weeks. The experimental groups consisted of 51 HH oysters (3% tank mortality), 48 HL oysters (4% mortality), 32 LH oysters (32% mortality), and 46 LL oysters (8% mortality). Mortality occurred sporadically, starting after 2 weeks of acclimation and continuing until the oysters were shucked for tissue sampling. For RNA-seq, 6 individuals per group were drawn randomly from archived tissues.

Reference Transcriptome

Given the availability of 2 published transcriptomes for *C. virginica* at the time of this work (Eierman and Hare 2014; Zhang et al. 2014), each developed from different life stages, we chose to combine them to gain a more comprehensive reference. The transcriptomes were combined using Cd-hit-est v.3 (Li and Godzik 2006). The goal was to reduce the redundancy of sequences in the 2 transcriptomes without reassembling from raw reads. Following the recommendations of Eierman and Hare (2014), Cd-hit-est was implemented using a sequence identity threshold of 0.8 and a word size of 5, collapsing the combined 116 965 reference sequences into 73 220 clusters. The clustered sequences were then re-annotated as described in De Wit et al. (2012), using the *C. gigas* protein set downloaded from NCBI (on 15 January 2014). The full NCBI nr database was not used because 89.2% of the annotated sequences from Eierman and Hare (2014) and 99.2% of the 48 562 sequences annotated by Zhang et al. (2014) were annotated from *C. gigas* proteins. The combined reference transcriptome sequences are referred to as "contigs" hereafter, with the longest sequence per Cd-hit-est cluster used to represent that cluster.

RNA-seq Library Preparation, Sequencing, and Quality Filtering

The mRNA was extracted using Dynabeads® mRNA DIRECT™ Purification Kit (Ambion®) and purified using the RNeasy® MinElute® Cleanup Kit (Qiagen®). The mRNA quality was assessed using an Agilent 2100 Bioanalyzer. The NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England BioLabs® Inc.) was used to prepare double-stranded cDNA libraries for sequencing with each individual barcoded. For PCR we used the KAPA Real-Time Library Amplification Kit (KAPABiosystems). The multiplexed libraries were sequenced on 5 lanes of 100 bp Hi-Seq Illumina 2000 at the Institute of Biotechnology at Cornell University. Reads were separated based on barcodes, clipped with fastz_clipper, and trimmed from either end up to any phred-scale quality score of >33 with fastq_quality_trimmer (FASTXToolkit). Reads shorter than 90 bp were discarded.

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying our analyses with Dryad.

Read Mapping and Differential Expression

We used Burrows-Wheeler Aligner (Li and Durbin 2009) parameters that maximized the number of reads that mapped uniquely and the number of contigs with mapped reads as described in Eierman (2014). We chose a maximum edit distance (*–n*) of 20 to correspond with the clustering similarity applied to merge the 2 published transcriptomes, and a maximum edit distance in the seed (*–k*) of 3. All other parameters were used at their default values to map each of the 24 samples against the reference transcriptome.

Differentially expressed genes were identified using the edgeR package (Robinson et al. 2010) implemented in R (R Development Core Team 2012). A file of read counts per contig for each sample was generated from BWA output using a custom script from De Wit et al. (2012). Each sample was identified as belonging to a reef (high or low) and treatment salinity (high or low). Read counts were normalized and "tagwise" dispersions (variance in read counts for each contig) were estimated in edgeR before fitting each contig to a GLM log-linear negative binomial model using the prior.count argument. To explain variation in expression levels, 3 factors were tested using separate likelihood ratio tests: 1) reef, 2) treatment, and 3) reef-by-treatment interaction (R × T). To further test for any reef-specific patterns of differential expression in response to treatment, we completed 4 planned contrasts, comparing, 4) both treatments for high-source oysters, 5) both treatments for low-source oysters, 6) both reef sources in the low salinity tank, and 7) both reef sources in the high salinity tank for a total of 7 likelihood ratio tests per contig. The resulting *P*-values from all 7 sets of likelihood ratio test comparisons were corrected for multiple comparisons using the false discovery rate (FDR) as described by Benjamini and Hochberg (1995), and contigs with a FDR <0.05 were considered significantly differentially expressed.

WEGO (Ye et al. 2006) was used to explore the gene functions, as identified by GO terms, for the differentially expressed contigs. The DE contigs represented a broad range of diverse functions. Because the relevance of GO terms for osmoregulatory function in the eastern oyster is unclear, we tested for functional enrichment of differentially expressed contigs using 3 custom-made functional groups from pertinent literature. The "osmoregulatory" functional group included 1241 candidate proteins experimentally identified in *C. gigas* using treatments of salinity from 5 to 40 on adults (Zhang et al. 2012). The "FAA" functional group included 22 free amino acid (FAA) metabolism proteins identified as osmoregulatory in *C. gigas* by Meng et al. (2013). Of these 22 FAA proteins, eleven were also identified as "osmoregulatory" candidates in the Zhang et al. (2012) study. The "stress" group included 112 proteins upregulated prior to summer mortality events in experimental *C. gigas* (Chaney and Gracey 2011). The "stress" and "osmoregulatory" functional groups (but not the "FAA" subgroup) overlapped by 30 proteins. Enrichment for functional groups, each identified from annotations at the protein level, was tested among differentially expressed (DE) contigs for each factor using Fisher's exact tests. Each tested the null hypothesis that the differential expression of contigs for a factor (DE vs. not DE) was independent of their membership in a specific functional group (member vs. nonmember) (n = 42 072).

SNP ID and Analysis

The Genome Analysis Toolkit (GATK version 2.8; McKenna et al. 2010; DePristo et al. 2011) was used to detect SNPs, following Broad Institute best practices and recommendations from De Wit et al. (2012) but without base quality score recalibration (http://www.broadinstitute.org/gatk/guide/best-practices downloaded February

2014). An initial set of variants was obtained using a phred-scale SNP quality threshold of 30 and a 10 bp window size in which clustered SNPs were evaluated during filtering. Then, a variant quality score threshold of 4 was used to train the variant quality score recalibration model. Genotypes called from the final set of SNPs were then filtered based on a phred-scale genotype quality threshold of 20.

Next, SNPs were restricted to those for which at least 20 individuals had confident genotypes, and we removed SNPs with a minor allele frequency less than 0.25 because they are the ones most subject to low coverage artifacts and generally have low information content (Roesti et al. 2012). Additional filtering removed contigs that had a combination of SNP density greater than 0.05 SNP/bp and heterozygosity greater than 70% at any one SNP because these patterns are expected to result from paralogous comparisons. All analyses used the fully-filtered SNPs. Nucleotide diversity, Wier and Cockerham F_{ST}, and linkage disequilibrium (LD), as measured by r^2, were then calculated using vcftools (Danecek et al. 2011). To test for SNPs under selection based on F_{ST} and the expected variance in F_{ST} from drift and sampling error, we used BayeScan v.2.0 (Foll and Gaggiotti 2008) with default parameter settings. For analyses at the level of exons we mapped contigs to the *C. gigas* genome using GMAP with the cross-species and slice-variant flags and the alignment and sam file output arguments (Wu and Watanabe 2005). Partial exons were included.

We then tested for evidence of selection at the DNA level by analzying the relationship between LD (pairwise allelic correlations among SNP loci, r^2) and nucleotide diversity, each measured at the level of exons within each reef, using a Pearson correlation test. Among the 627 outlier-containing contigs, 490 had exons uniquely mapped to the *C. gigas* genome. Out of a total of 5435 mapped exons, 70 had multiple SNPs including at least one classified as an outlier, and these were used to calculate r^2 within exons.

Results

Reference Transcriptome

The consolidation of the 2 transcriptomes by clustering resulted in 42 072 annotated contigs (see Supplementary Information online for further details). Of the 1241 osmoregulatory candidate proteins identified in *C. gigas*, defined here as the "osmoregulatory" functional group, 1036 were represented by 9785 contigs in the combined reference transcriptome. Of the 22 proteins for the "FAA" group defined in Meng et al. (2013), 15 were represented by 58 contigs. Finally, of the 112 proteins for the "stress" functional group (Chaney and Gracey 2011), 65 were represented by 402 contigs.

Illumina Read Mapping and Differential Expression

An average of 18 684 557 (66.4%) reads per barcoded sample (range: 7.2–35.7 million) remained after quality filtering. An average of 44.1% of the remaining reads mapped to the reference transcriptome, and of these, 93.7% mapped uniquely. Only uniquely mapped reads were used in our analysis. The average depth of coverage per sample after mapping, including zero depths, was 18.3 reads per bp.

A total of 9921 contigs (23.6%) were identified as significantly differentially expressed (DE) with a FDR <0.05 (Figure 1). Many contigs were significant for more than one factor so among all contigs, 0.6% showed a reef effect, 18.9% showed a treatment effect, and 13.9% showed a R × T interaction (Figure 1). Of the 252 contigs showing a reef effect, only 21 were significantly DE for the reef factor alone, with an average 3.2 $\log_2$-fold difference in expression

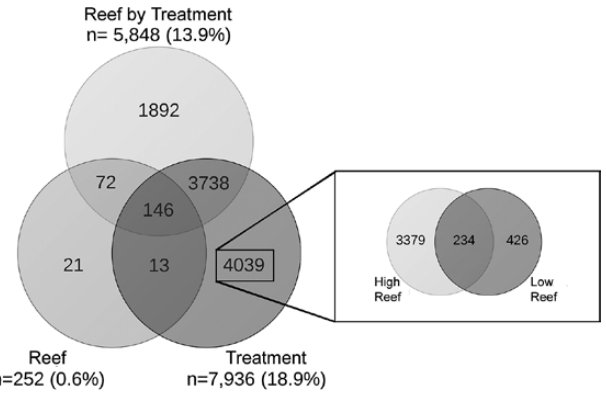

Figure 1. Venn diagram of the number (and proportion) of contigs that were differentially expressed for each factor out of 42 072 contigs. Overlaps indicate that the gene was differentially expressed in response to multiple factors. The center indicates that 146 contigs were differentially expressed in response to reef source (Reef), treatment salinity (Treatment) and the interaction between reef source and treatment salinity (R × T). The treatment-only contigs were further investigated by contrasts within each reef and the boxed Venn diagram shows these results. Of these 4039 DE contigs, 234 were differentially expressed in response to treatment by both reefs whereas 3379 were significant for a treatment response only in the high reef and 426 were significant only in the low reef.

between the 2 reefs but no significant plasticity across treatments. In comparison, out of 7937 contigs significant for a treatment effect, 4039 were strictly so with no significant effect from other factors. Finally, for a total of 5848 contigs the response to treatments was different in the 2 reefs and for a subset of 1892 this was the only significant factor (R × T; Figures 1 and 2A, C). We examined 2 model contrasts to determine if contig expression differences between reefs were greater in one treatment or the other (LL vs. HL and LH vs. HH). There was nearly 15 times more DE contigs between reefs after acclimation to low salinity (3675 contigs) than in the high salinity treatment (252 contigs).

Contigs showing expression variation attributed solely to a treatment effect, classical plasticity with both reef populations changing expression in the same direction, might be expected to show similar degrees of plastic response in both populations, or more plasticity expressed by the low-salinity reef oysters because their developmental environment was more variable (see Supplementary Figure S2 online). We used 2 reef-specific contrasts to measure expression plasticity in each reef separately (HH vs. HL and LH vs. LL). Only 234 contigs showed a significant treatment response in both reef populations (e.g., Figure 2D), resulting from similar fold-change responses in the 2 populations and reflected in their position along the treatment-effect volcano plot diagonal (Figure 2B). The remaining 3805 treatment-only DE contigs responded significantly to treatment in only one of the 2 reefs due to an asymmetry in the average steepness of their reaction norms. The high salinity reef oysters had 3379 contigs DE between treatments (88.8% of all treatment-only DE contigs; Figure 2B,E) compared to only 426 contigs DE (10.5%) for the low salinity reef oysters (Figure 2B,F). Accordingly, the high salinity reef oysters had significantly steeper reaction norm slopes, measured as the absolute $\log_2$-fold change in fitted read counts for each contig between treatments, than did the low salinity reef oysters (Wilcoxon signed rank test, $P < 0.0001$, Figure 3). Both populations more frequently responded with up-regulation when confronted with the "away" treatment, but the magnitude of this asymmetry was much greater for the low salinity reef (Table 1, Fishers exact

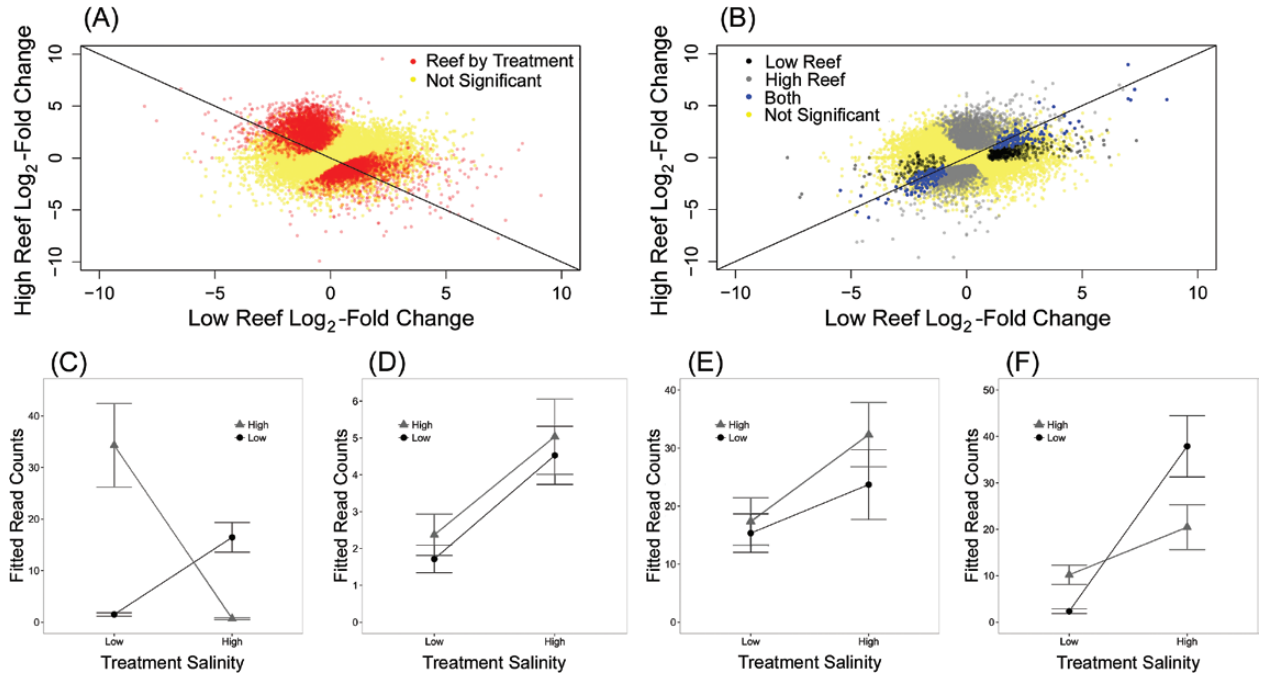

Figure 2. Differentially expressed contigs with highlighting of those significant only for (**A**) R × T interaction or (**B**) Treatment factor. Each point represents a single contig. In (A) and (B) 32 151 contigs that are not differentially expressed for any factor are presented as background points. In (A) there 1892 contigs significant for the R × T interaction. In (B) there are 234 contigs significant for the treatment factor in both reefs, 426 contigs for the low reef, and 3379 contigs for the high reef. Each axis indicates the $\log_2$-fold change in expression in response to the high salinity treatment compared to the low salinity treatment for each reef. The diagonal line represents the expectation for the factor (R × T or Treatment) represented in the graph. (**C**–**F**) are example reaction norms for single points in the scatterplots. (C) is a "G × E" reaction norm for a significant point in (A); (D), (E), and (F) are "plasticity" reaction norms for both, high reef and low reef, respectively, in (B). The reaction norms depict read counts that have been normalized and fit to a negative binomial model. Mean read counts are plotted separately for each reef source. Error bars are standard error.

test, $P < 0.001$). The high salinity reef oysters had 17% more up-regulated contigs in response to the "away" (low salinity) treatment relative to their "home" treatment. For the low reef oysters, more than 3 times as many contigs were significantly up-regulated in the "away" (high salinity) treatment than at low salinity (Table 1).

Statistical tests for functional enrichment within mutually exclusive groups of significantly DE contigs were generally not significant. The only evidence of functional enrichment was for the "stress" group in contigs differentially expressed for the treatment-only factor (n = 42 072, P = 0.00023, odds ratio = 1.72). Looking at contigs showing significant treatment DE in each reef based on contrasts, enrichment for "stress" genes was found both for DE contigs unique to the low reef oysters (P = 0.0086, odds ratio = 2.52) and those unique to the high reef oysters (P = 0.0097, odds ratio = 1.52). The few contigs DE for both reefs were not significantly enriched (P = 0.075, odds ratio = 2.28). Similar to the overall expression patterns for treatment-only DE contigs (Table 1), up-regulation of "stress" contigs in the high salinity reef oysters was only slightly more common in the "away" treatment (22 contigs upregulated at "home" and 26 "away") but a much larger proportion were up-regulated in the "away" treatment for the low salinity reef oysters (1 "home" and 10 "away").

SNP Patterns

A total of 1 345 639 SNPs were identified using GATK Unified Genotyper. After quality filtering, 79 660 SNPs remained in 12 240 contigs, and the mean F_{ST} between reefs was 0.0025 (Figure 4). Model-based locus-by-locus BayeScan tests found no SNPs that appeared to be under selection, likely due to low power from small sample sizes (FDR threshold = 0.1). Nonetheless, there were many highly differentiated loci. The 797 SNPs above the 99th percentile of F_{ST} values, located within 627 contigs, had F_{ST} values ranging from 0.29 to 0.73 and were provisionally defined as outliers for further analyses (Figure 4). Using a permutation test to randomly re-assign the 24 samples to 2 populations and generate a null distribution of 99th percentile F_{ST} values, we found that our empirical 99th percentile F_{ST} cut-off value of 0.29 for defining outliers was significantly higher than the 99th percentile values (mean = 0.28) expected by chance (P = 0.036), supporting the statistical categorization of these SNPs as potentially non-neutral outliers. A list of outlier-containing contigs and their annotations is given in Supplementary Table 1 online. Of these outlier-containing contigs, 107 (17%) contained multiple outlier SNPs (277 SNPs total).

We hypothesized that if outlier SNPs were clustered within contigs because of differential viability selection on haplotypes, then selection would have increased the LD between SNPs while lowering the within-reef nucleotide diversity. Similarly, when analyzing the combined reefs, we hypothesized that disruptive selection would have generated a positive relationship between LD and nucleotide diversity. We first explored the relationship between LD and the distance between SNPs (see Supplementary Information and Supplementary Figure S3 online), and based on the rapid decline of LD beyond 100 bp, we focused our analysis at the level of exons. The low salinity reef source oysters had a significant negative correlation between exon-level r^2 and nucleotide diversity (Pearson correlation test, P = 0.019, rho = –0.28, Figure 5) whereas the same set of exons showed no correlation in the high salinity reef source oysters

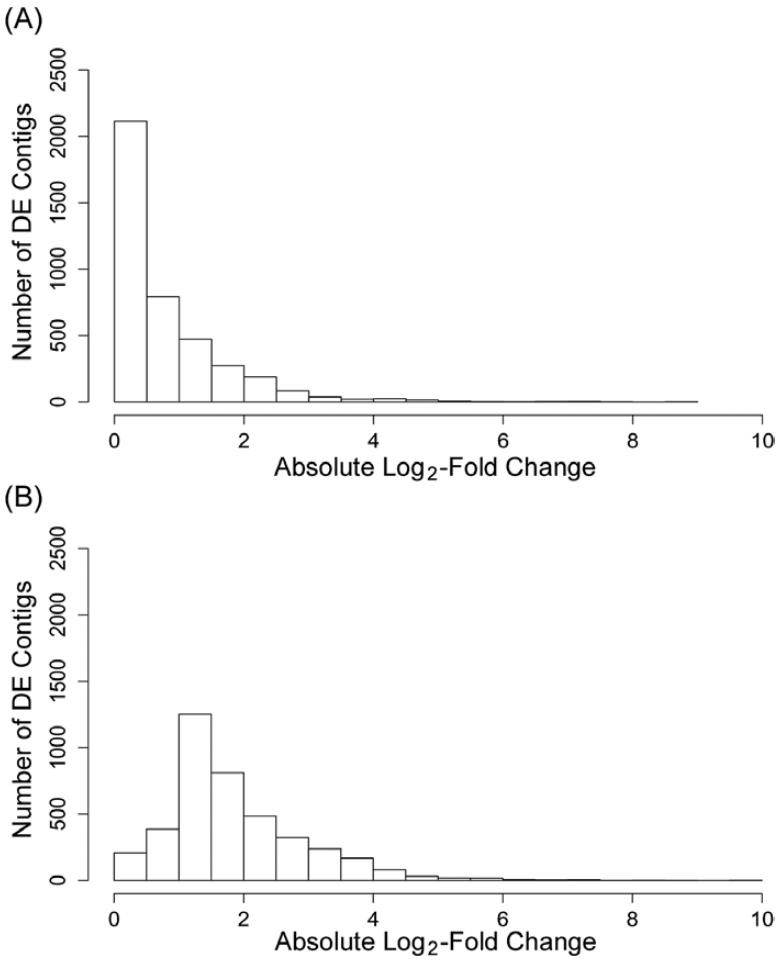

Figure 3. Distributions for the absolute values of the $\log_2$-fold changes for contigs significantly DE for only the Treatment factor. The $\log_2$-fold change is proportional to the slope of a reaction norm between the low and high treatment salinities. A steeper slope in a reaction norm is a larger $\log_2$-fold change. (**A**) The distribution for the low salinity reef oysters. (**B**) The distribution for the high salinity reef oysters.

Table 1. Treatment-only differentially expressed contigs that are significant in only one reef source

	High salinity reef (n = 3379)	Low salinity reef (n = 426)
Low salinity tank	1825	98
High salinity tank	1554	328

The values indicate the number of contigs that had significantly higher expression in the associated tank compared with the alternative tank.

(P = 0.72, rho = 0.042), suggesting stronger selection on the low-salinity reef. Average nucleotide diversity in these outlier-containing exons was significantly different in the low- (0.34) and high-salinity reef (0.41) oysters (paired t-test, P = 0.035). The outlier-containing contigs also had 384 exons without outlier SNPs and for these average nucleotide diversity was not significantly different between the 2 reef sources (low = 0.459 and high = 0.455; P = 0.23). Testing for an association in the combined reef sample showed a nonsignificant trend for positive correlation between LD and nucleotide diversity (Pearson correlation test, P = 0.077, rho = 0.21) consistent with the hypothesis of disruptive selection between the 2 reefs.

In order to determine if outlier SNPs in coding sequences of transcripts were disproportionately associated with significantly DE contigs, as might be expected if selection acted jointly on regulatory and coding sequence variation or if linkage created an association, we tested for the enrichment of DE contigs within the set of 627 contigs containing outlier SNPs in comparison to the remaining 11 613 SNP-containing contigs that did not contain an outlier SNP. The 2 experimental factors and the R × T interaction term were tested separately (number of contigs with SNPs: reef: n = 9, treatment: n = 2006, reef by treatment: n = 922) and none showed significant enrichment of outlier SNPs within DE contigs. Because spatially variable selection on coding sequences might be expected for osmoregulatory or stress related genes in the estuary, we also tested for enrichment of "osmoregulatory" (n = 2170 contigs with SNPs), "FAA" (n = 21), and "stress" (n = 140) contig annotations within the set of contigs containing outlier SNPs. The only significant test indicated enrichment for the "FAA" group (P = 0.020, odds ratio = 4.38). Outlier SNPs were in 4 of the 21 polymorphic "FAA" contigs: aldehyde dehydrogenase family 3 member B1 (10 outlier SNPs), cysteine dioxygenase type 1 (1 outlier SNP), argininosuccinate lyase (1 outlier SNP), and pyrroline-5-carboxylate reductase 2 (1 outlier SNP). The aldehyde dehydrogenase family 3 member B1 contig, in addition to having multiple coding sequence SNPs with high F_{ST} values between the reef samples (see Supplementary Information and Supplementary Table 2 online), showed significant DE for the R × T interaction factor.

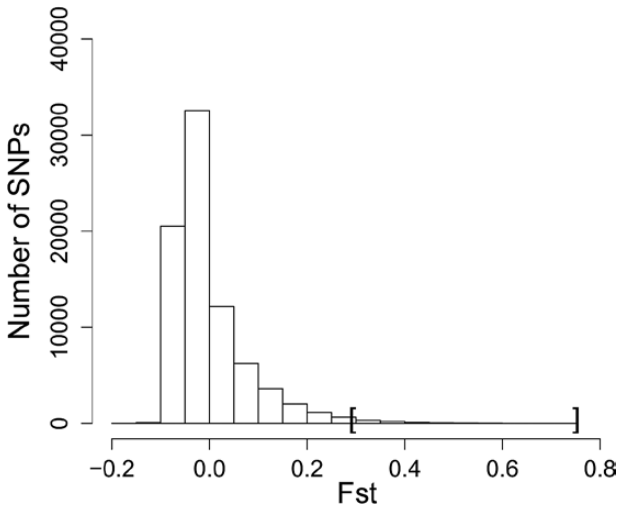

Figure 4. Distribution of Wier–Cockerham F_{ST} values for all SNPs with minor allele frequency greater than 0.25. Range of F_{ST} values treated as outliers shown with bracket.

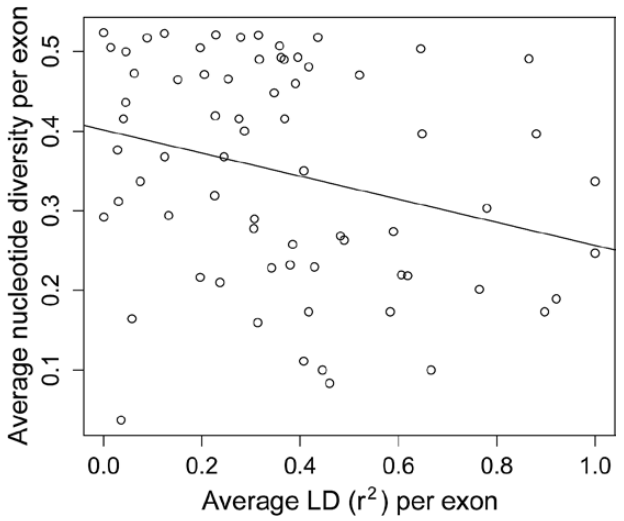

Figure 5. From the set of 107 contigs that contained >1 outlier SNPs, 70 exons containing at least one outlier SNP are shown with respect to exon-level LD (r^2) and nucleotide diversity for the low salinity reef source oysters: The line shows a significant negative correlation (Pearson correlation; $P = 0.019$) with a rho value of −0.28.

Discussion

Our results show both highly plastic and reef-specific patterns of gene expression in *C. virginica* after acclimation to different osmotic conditions. A total of 7936 contigs, representing 5669 genes and coding for 4818 proteins, were differentially expressed across treatments, demonstrating genomically pervasive plastic responses to salinity variation. Plasticity in response to osmotic pressure also involved many genes in *C. gigas*. In pairwise experimental comparisons of salinities ranging from 5 to 40, *C. gigas* had a total of 1761 differentially expressed genes coding for 1241 proteins (Zhang et al. 2012). Similarly, Zhao et al. (2012) identified 3480 *C. gigas* contigs that responded to salinity variation. Indeed, gene expression plasticity involving many genes has repeatedly been shown in species maintaining physiological homeostasis across environments (e.g., Smith et al. 2013; Cheviron et al. 2014; Côté et al. 2014). Differential expression may be genomically pervasive because of strong secondary effects in response to changes in the primary osmoregulatory factors, or as a result of generalized stress responses in conjunction with homeostatic responses (de Nadal et al. 2011; Zhang et al. 2012; Luhua et al. 2013).

Given that the 2 sampled reefs seem to share high gene flow as indicated by low genetic differentiation (total genomic mean $F_{ST} = 0.0025$; non-outlier SNPs mean $F_{ST} = -0.0044$) and previous studies using neutral markers (He et al. 2012), it is remarkable that the plasticity exhibited by oysters from the 2 reef sources differed so much in pattern and magnitude. Interestingly, almost all reef differences were context dependent. Relatively few contigs showed differential expression between reefs based solely on the reef factor, independent of treatment environment. In contrast, adult oysters collected at high and low salinity reefs showed opposite gene expression responses across treatments (reaction norm slopes) with significant reef source-by-treatment interaction at 5898 contigs, representing 4427 genes coding for 3811 proteins. In addition to these G × E contigs showing a different pattern of salinity response between reefs, the treatment-only DE contigs provided evidence for different magnitudes of plastic response in oysters from the 2 reefs. The median among-locus fold change in expression for the treatment-only contigs was 71% smaller in the low salinity reef oysters, as reflected by shallower slopes for reaction norms, than in the high salinity reef oysters. Consequently, oysters from the low-salinity reef also had 8-fold fewer contigs that were statistically significant for differential expression across treatments, compared with oysters from the high-salinity reef. These results indicate that oysters from these 2 reefs had fundamentally different gene regulatory "solutions" after surviving the natural conditions at their reef and then acclimatizing to osmoregulatory challenges in our experiment. Despite the fact that the low-salinity reef oysters inhabit a region of the estuary with slightly greater monthly variance in salinity (see Supplementary Figure S2 online), there was reduced plasticity in their gene expression in response to salinity treatments. In addition, for both reef sources the gene regulatory response to treatments included stress response genes, contributing to gene expression differences that were much greater between the reefs at 10 salinity than at 30. The higher salinity is closer to the range of salinities considered ideal for adult oysters (Swannack et al. 2014), so it seems that the more stressful treatment elicited greater differences in gene expression between oysters from the 2 reefs.

Two mechanisms capable of producing these reef specific patterns are developmental plasticity and recurrent within-generation differential viability selection. Developmental plasticity is typically considered irreversible and often applied to morphological traits. However, to the extent that acclimatization early in development (e.g., larval or early post-settlement) activates permanent within-generation epigenetic control over physiological responses, traits may show patterns of plasticity in adults that are only partially reversible. Examples of labile traits showing some plasticity canalization include differences in de-acclimation rate/success when warming follows acclimation to cold temperature (plant hardiness; Kalberer et al. 2006; Gorsuch et al. 2010). Our experimental acclimation of oysters over 9 weeks is likely sufficient to reset any reversible plasticity, but developmental epigenetic mechanisms and trans-generational epigenetic effects controlling trait variation could have persisted and confounded genetic effects.

If the individuals taken from both reefs were a random sample from an overall homogeneous and genetically diverse population

within the estuary, then no G × E or reef effect should be detected beyond that from sampling error. The large number of contigs showing a G × E interaction after acclimation suggests that the samples collected from high and low salinity reefs represent functionally and genotypically differentiated populations. The gene expression patterns that accomplish cell volume homeostasis were very different in the 2 populations, particularly when compared at low salinity. These differences in gene expression patterns are consistent with physiologically divergent phenotypes that resulted from differential viability selection across the estuarine salinity gradient. The degree to which this evolutionary mechanism operates recurrently within generations versus cumulatively over many generations depends on the pattern and magnitude of gene flow within the estuary, the strength of selection, and the interaction of these 2 forces with genetic drift.

We previously reported salinity tolerance experiments on larval cohorts produced from the adults described here (Eierman and Hare 2013). We hypothesized that oysters from low and high salinity reefs would produce larvae with reef-specific levels of salinity tolerance. To test this we measured larval survival to 14 days for multiple families exposed to different salinity treatments. Larvae from the high salinity reef showed similar survival across salinities of 10 and 30 whereas larvae from the low salinity reef had greatly reduced survival in the high salinity treatment. The larval results mirror adult gene expression patterns in that offspring from the low-salinity reef adults showed a narrower range of tolerance than did larvae from the high salinity reef, perhaps reflecting the shallower reaction norm slopes of the low salinity reef adults. Thus, aspects of population differentiation between reefs were found to be heritable, but transgenerational epigenetic effects cannot be ruled out.

Inferences of Selection at the DNA Level

Given that logistically infeasible experimental conditions are necessary to completely rule out epigenetic effects, we attempted to infer differential viability selection based on DNA sequence differentiation between the reefs. Even though differential gene expression is controlled by gene regulatory variation outside of transcript coding sequences, within-generation selection for distinct phenotypes across habitat heterogeneities might also cause differentiation of coding sequence frequencies at relevant genes. We found that patterns of linkage disequilibrium and nucleotide diversity, as well as functional enrichment within the outlier SNP-containing contigs, supported the inference that selection shaped some patterns of differentiation between reefs at some loci.

Specifically, we reasoned that if selection is on haplotypes instead of individual SNPs and is strong enough, then elevated LD within reef samples should be negatively correlated with nucleotide diversity. Measuring average pairwise r^2 at the level of exons, and applying the test to contigs containing one or more outlier F_{ST} SNPs, a significant negative correlation was present only in the low salinity reef, suggesting stronger recent selection at that location. In addition, contigs with F_{ST} outlier SNPs occurred more frequently within the FAA functional group than expected by chance based on the overall frequency of outlier-containing contigs. The FAA functional group is small (21 polymorphic contigs) and contains candidate genes with the strongest empirical support for osmoregulatory function in Crassostreid oysters (Meng et al. 2013). Together these patterns suggest that selection, particularly on the low salinity reef, is contributing to functional genetic differentiation between the reefs even while most of the genome remains homogenized by gene flow. Given the oyster's life history, including astounding fecundity and punishing early mortality, it is likely that most of the genetic differences between reefs are a consequence of recurrent within-generation selection. Cohort analyses by Yu and Guo (2005) have previously demonstrated large within-generation changes in allele frequencies at a single Delaware Bay site where associations with Dermo disease pressure led to inference of disease resistance/tolerance QTLs (see also Hofmann et al. 2009). However, we cannot exclude the possibility that multigenerational adaptation of the up-bay oysters contributes to the observed differences.

Our ability to test for F_{ST} outliers was restricted by low power and the inherent sensitivity of such tests to transcriptome SNP artifacts from paralogs and variation in depth of sequencing coverage. We identified candidate non-neutral outliers using the top 1% of the F_{ST} distribution. Given the small sample size, we supported this result using a permutation test to indicate that such a skewed tail is unlikely as a function of sampling error. To prevent genotyping error due to paralogs, we applied stringent filtering to the SNPs including the removal of any contigs with SNPs at high density, and removal of SNPs with heterozygosity ≥70%, or MAF <0.25. Finally, we repeated outlier SNP analyses after removing the 27.6% of SNPs that had statistically unequal coverage between the 2 reefs. All statistical conclusions remained the same, including the LD versus nucleotide diversity correlation in the low salinity reef oysters. These analytical attempts to filter out potential biases seem to have provided a robust demonstration of selection at the DNA level despite small sample sizes. It will be important to follow-up with larger sample sizes, more direct measures of spatial allele frequency differentiation at the DNA level, and within-generation tests of allele frequency change within cohorts.

Comparison of Plasticity Patterns to Similar Studies and Theory

The gene expression patterns reported here suggest that a combination of plasticity and recurrent within-generation selection has led to the evolution of a broad realized salinity niche in Delaware Bay oysters. Most assessments of the relative importance of plasticity and natural selection in response to climate change have focused on classical intergenerational selection driving evolutionary change, and therefore dichotomized these responses into short-term and long-term. The impact of selection on plasticity evolution has been investigated primarily with respect to irreversible developmental plasticity, often in the low gene flow context of adaptive evolution (Crispo 2008; Fitzpatrick 2012; Scheiner 2013). Developmental plasticity is often assumed to be relatively low in fitness cost, but its evolution is strongly influenced by environmental predictability during the temporal gap between a developmental switch and environmental selection later in life (Lande 2009). By contrast, labile traits have plastic expression in sync with temporal changes in environment. For labile traits the magnitude of evolved plasticity still depends on environmental variability and predictability, but is more heavily influenced by the costs (e.g., metabolic) of plasticity (Lande 2014).

If the distinct patterns of gene expression between oyster reefs diverged as a result of within-generation selection, how do the differences in patterns of plasticity accord with existing theory on the evolution of plasticity? Between the 2 reef populations studied here, we suppose that the "high" salinity reef has plasticity patterns closer to the species "norm" because 30 salinity is close to the optimum salinity range for *C. virginica* (Swannack et al. 2014) and evidence for selection on the high salinity reef was absent. The within-generation evolution of a distinct plasticity pattern at the low salinity reef

may be a relevant context in which to apply a recent model describing transient phases of plasticity evolution after a sudden shift to an extraordinary environment (Lande 2009, 2014). With respect to labile traits, Lande's model (2014) predicts a sudden and transient increase in plasticity after a shift to an extraordinary environment, with larger increases for more variable or predictable environments or when plasticity costs are lower relative to the strength of stabilizing selection on the character. If we consider the low salinity reef as populated by larvae primarily generated from higher density oyster populations at higher salinity, then the reduced plasticity observed in the low salinity reef oysters is counter to the trend expected after movement to an "extraordinary" environment and may be indicative of a larger relative cost of plasticity in the low salinity region of the estuary. Higher costs could be incurred as a result of antagonistic pleiotropy such that the locally competitive genotypes with high post-settlement survivorship in marginal environments have "traded" metabolic efficiencies for their ability to tolerate low salinity. It is noteworthy that two-thirds of the osmoregulatory candidates identified by Zhang et al. (2012) in *C. gigas* also were differentially expressed in response to other independently applied stressors, including air exposure, temperature, and heavy metals. Given this overlap in environmental triggers, it is likely that a large subset of the genes responding to salinity treatments in this study is pleiotropic.

An alternative hypothesis is that marginal environments impose recurrent within-generation selection for genotypes that are resilient to stress. Studies involving experimental gene expression comparisons similar to those described here between environmentally typical and marginal populations have highlighted subsets of genes in the more resilient population that have constitutively higher expression, perhaps pre-acclimating them to stress, while other genes have constitutively lower expression, as if less response is needed to maintain homeostasis (Pearson et al. 2009; Zippay and Hofmann 2010; Barshis et al. 2013). This resilience perspective is not a good match for the Delaware Bay oysters if we consider the low salinity reef to be marginal with respect to ideal salinity variation (Swannack et al. 2014). We found an overall lower level of expression in the hypothetically "tolerant" low-salinity population, but they were not more resilient. The low-salinity adult oysters had twice the tank mortality as did high-salinity oysters when each was in their home treatment (8% vs. 4%), and in the "away" treatment the low salinity oysters suffered 32% mortality. Furthermore, oysters from the low salinity reef produced less tolerant larvae (Eierman and Hare 2013). Stress and the evolved or plastic response to stress are certainly critical factors to understand in these oysters, but so far the patterns do not fit neatly into recent conceptualizations for the distribution of resilience across populations.

Mechanisms Contributing to Variation in Plasticity

Natural selection may have acted to generate these G × E patterns in gene expression through several different, but not mutually exclusive processes. First, the many parallel metabolic pathways that bivalves use for osmoregulation (Evans 2009; Meng et al. 2013) may make this trait a particularly variable target for selection. Osmoregulation in bivalves is a meta-trait involving many metabolic pathways for controlling the flux of organic osmolytes, but it may also involve a diversity of gene networks because of gene duplication. In *C. gigas*, genes differentially expressed in response to stressors such as salinity had a greater frequency of paralogs compared with humans and sea urchins (Zhang et al. 2012). As reviewed by Conant and Wolfe (2008), duplicated genes may be co-opted for "new" functions, although often times these functions are a secondary property that was present in the original copy. In many cases the neofunctionalization of duplicate genes involves the divergence of context-specific expression (e.g., McConn et al. 1994; Yurchenko et al. 2014). Gene networks that include a higher proportion of gene duplicates may provide more variation among genotypes in the genetic architecture for plasticity of labile traits, leading to habitat-specific gene expression patterns in response to selection across habitat heterogeneities.

So far we have interpreted these experimental results with an assumption of completely homogenizing gene flow such that evolutionary change from selection must be confined to within-generation transient patterns. To the contrary, models of larval dispersal in the Delaware Bay (Narváez et al. 2012) and Chesapeake Bay (North et al. 2010) predict an asymmetry in the advection of larvae such that the up-bay regions get few larvae immigrating from down-bay reefs while the down-bay regions get recruits from a more well-mixed pool. Specifically in a Delaware Bay model (Narváez et al. 2012), 50% of larvae from low salinity were predicted to disperse down-bay whereas ~1% of the larvae from high salinity reefs dispersed up-bay. This asymmetry may allow genotypes in the low salinity reef to experience some measure of trans-generational adaptation to hypo-osmotic conditions. In contrast, the genotypes settling in the down-bay regions come from many source populations, perhaps accumulating a greater amount of functional genetic diversity, and selection under such high gene flow will only spatially alter allele frequencies within single generations. It will be important to model plausible dispersal asymmetries under different quantitative trait models for plasticity to explore the relative importance of within-generation and between generation evolutionary processes for generating the observed plasticity differences.

Conclusions and Relevance to Oyster Restoration

Overall, the findings of genomically pervasive plasticity along with extensive G × E effects have implications for how organisms with high early mortality and high gene flow will acclimatize and adapt to a rapidly changing environment. By extension from the non-neutral SNP patterns, much of the observed gene expression divergence between reefs is hypothesized to be a result of recent selection they experienced in the wild. In the context of high gene flow this divergence suggests that intra-generational selection created a patchwork of functionally distinct adult genotypes and widened the species' realized niche. Studies on the interaction between plasticity and environmental change typically focus on selection acting across multiple generations to result in local adaptation (e.g., Carroll et al. 2007; Lande 2009; Reed et al. 2011; Raubenheimer et al. 2012). While this focus may be appropriate for most species, we suggest that for many plant and marine benthic species intragenerational selection molds patterns of plasticity across habitats and needs to be taken into account to understand the diversity of reaction norms and their adaptive value. These insights have immediate consequences for oyster restoration practice because they suggest that phenotypic plasticity cannot be counted on to achieve high mean fitness for the offspring of hatchery broodstock regardless of broodstock origin or outplant location of their progeny.

The within-generation selection inferred here may seem unimportant as a vehicle leading to lasting evolutionary change if gene flow spatially randomizes each larval cohort. However, strong selection makes the classical balance between gene flow and selection more sensitive to gene flow asymmetries or moderate but persistent gene flow barriers (Lenormand 2002). Thus, nonrandom patterns of

larval dispersal within estuaries may have more evolutionary influence than expected based on neutral marker phylogeography (Rose et al. 2006). More importantly, the high levels of standing genetic variation that evolve with a Type III life history, including variation in plasticity in this case, may be a key trait that accelerates evolutionary responsiveness to environmental change (Romiguier et al. 2014).

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/

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