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SYMPOSIUM

Evolutionary Change in the Eastern Oyster, *Crassostrea Virginica*, Following Low Salinity Exposure

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Synopsis The presence of standing genetic variation will play a role in determining a population's capacity to adapt to environmentally relevant stressors. In the Gulf of Mexico, extreme climatic events and anthropogenic changes to local hydrology will expose productive oyster breeding grounds to stressful low salinity conditions. We identified genetic variation for performance under low salinity (due to the combined effects of low salinity and genetic load) using a single-generation selection experiment on larvae from two populations of the eastern oyster, *Crassostrea virginica.* We used pool-sequencing to test for allele frequency differences at 152 salinity-associated genes for larval families pre- and post-low salinity exposure. Our results have implications for how evolutionary change occurs during early life history stages at environmentally relevant salinities. Consistent with observations of high genetic load observed in oysters, we demonstrate evidence for purging of deleterious alleles at the larval stage in *C. virginica*. In addition, we observe increases in allele frequencies at multiple loci, suggesting that natural selection for low salinity performance at the larval stage can act as a filter for genotypes found in adult populations.

Introduction

For many marine organisms, current rates of dispersal and plasticity will not be sufficient to keep pace with predicted rates of ocean change, such as increased temperatures, acidification, and precipitation (Sunday et al. 2015). Persistence will therefore depend on the species' capacity for evolutionary adaptation (Hoffmann and Sgrò 2011). For metazoans, adaptation to current climate change is unlikely to occur from new mutations due to the fast rate of environmental change (Orr and Unckless 2008), but preexisting genetic variation among populations may provide the raw material necessary for evolution to occur (Barrett and Schluter 2008; Pespeni and Palumbi 2013). Genetic variation among populations can arise and be maintained by strong selective pressures imposed by environmental gradients, such as temperature and salinity, resulting in locally adapted populations (Kawecki and Ebert 2004; Sanford and Kelly 2011; Kelly et al. 2013). Alternatively, variation may be maintained through balanced polymorphisms within populations, usually when there are high levels of gene flow combined with balancing selection imposed by fine-scale environmental heterogeneity or negative frequency dependent selection (Schmidt et al. 2000; Bay and Palumbi 2014; Silliman 2019). As a result, species with broad geographic ranges and species that inhabit heterogeneous environments may harbor the genetic diversity for natural selection to act on and promote adaptation to future climate change (Alberto et al. 2013).

Evidence for rapid genetic adaptation to climate change stressors has been documented in many species. For example, shifts in allele frequencies in response to seasonal fluctuations in temperature, extreme heat

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waves, and gradual warming have been observed in *Drosophila* (Umina 2005; Rodríguez-Trelles et al. 2013; Bergland et al. 2014). Similar shifts in standing genetic variation have also been documented in marine organisms, such as sea urchins and mussels, that could allow them to adapt to emerging climate change stressors such as ocean acidification (Bitter et al. 2019; Brennan et al. 2019). Recent advancements in sequencing technologies provide the ability to measure genetic variation and detect shifts in this variation in response to climate change stressors, allowing us to more accurately assess population resiliency.

A species of particular concern is the eastern oyster, *Crassostrea virginica*, a widespread euryhaline species that is the center of a major fishery and aquaculture economy in the Gulf of Mexico, and also provides many environmental services, such as improving water quality, stabilizing coastlines in the Gulf of Mexico (Meyer et al. 1997), and providing a habitat for other fishery species (Plunket and La Peyre 2005). A major threat to eastern oysters in the northern Gulf of Mexico is the decline in estuarine salinities due to increases in precipitation during peak temperatures in the summer and fall (Swenson 2003; Powell and Keim 2015; Prein et al. 2017). More urgently, productive oyster breeding and aquaculture grounds are threatened by anthropogenic alterations to local hydrology. Efforts to protect the coastline from erosion will result in large-scale (~7000 m^3 s^{-1}) diversions of the Mississippi river, which will dramatically increase freshwater flow into oyster habitats (Wang et al. 2017). While *C. virginica* naturally experiences a range of salinity conditions from 4 to 35 psu, they can only withstand low salinity for short periods of time, with growth and competitive ability ultimately compromised, especially in the early life stages (La Peyre et al. 2013). Although the species seems to be highly plastic (Jones et al. 2019; Marshall et al. 2021), there is also evidence that salinity tolerance has a genetic component that may enable rapid adaptation (McCarty et al. 2020; Griffiths et al. 2021).

Crassostrea virginica face another challenge at the larval stage: the purging of deleterious alleles inherited from each parent and mutations accumulated during meiosis (Plough 2016). The mutation rate in the Pacific oyster, *Crassostrea gigas,* was recently estimated to be two to three orders of magnitude larger than any other reported eukaryote (Churches et al. 2021). Large genetic loads in highly fecund species may be explained by the large number of cell divisions, and opportunity for mutation, that occur during the production of millions of eggs or sperm each spawning cycle. Early evidence for this phenomenon in oysters came from distorted Mendelian segregation ratios of single-pair crosses as a consequence of differential mortality of individuals homozygous for recessive deleterious alleles (Launey and Hedgecock 2001; Li and Guo 2004). Highly fecund species, such as oysters, often experience high mortality during the larval phase, which may be caused by a combination of genetic load, environmental factors, and predation (Rumrill 1990). In the future, larvae are expected to be exposed to multiple sources of selection, such as low salinity exposure from climate change as well as purifying selection to remove highly deleterious alleles.

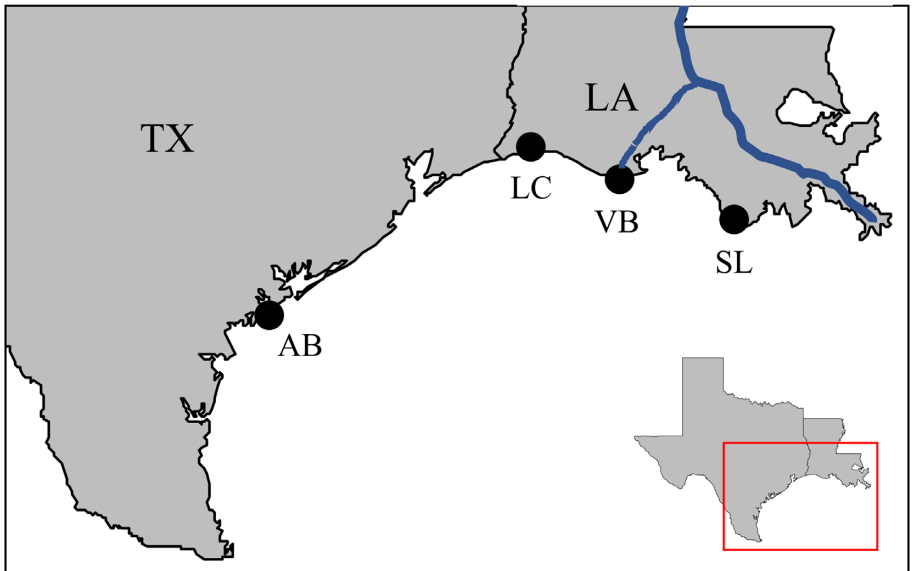
In this study, we quantified the presence of standing genetic variation in *C. virginica* larvae from both Texas and Louisiana populations, imposed by a strong low salinity selection event and high genetic load. We hypothesized that genetic variation for survival at low salinities is present within each population and that selection will result in changes to allele frequencies for genes associated with salinity tolerance. The substantial body of work on salinity tolerance in oysters, and the publicly available *C. virginica* genome, sets the foundation for the use of a targeted sequencing approach (e.g., exome capture) on genes that have previously been associated with low salinity tolerance in oysters. We observed changes in allele frequencies in these ecologically relevant genes, suggesting that *C. virginica* populations maintain standing genetic variation to combined low salinity selection and high genetic load.

Methods

Oyster collection and selection experiment

Oysters were collected from estuaries in Louisiana and Texas, which span the salinity gradient observed in the Gulf of Mexico. Louisiana oysters originated from a spawn that occurred at the Louisiana Department of Wildlife and Fisheries Michael C. Voisin (MCV) Oyster Hatchery located in Grand Isle, LA (29°14′17.98″N, 90°00′09.98″W). Broodstock used in this hatchery were collected by dredging from nearby populations in coastal Louisiana (Fig. 1). However, hatchery records on which population was used as broodstock to generate the parents used in our experiment were lost after spawning but would have originated from one of three potential source populations (Fig. 1). Texas oysters were collected from Aransas Bay (28°01′51.7″N, 96°59′53.2″W; Fig. 1). Salinities for Louisiana sites were calculated as daily averages from June 1, 2006, to June 1, 2016 (United States Geological Survey and Louisiana Department of Fish and Wildlife). Salinity for Aransas Bay was calculated as biannual averages from 2006 to 2016 (Texas Parks and Wildlife Department fisheries; Martinez-Andrade et al. 2018).

We used single-pair crosses to identify sources of genetic variation for low salinity performance.



Site	Latitude	Longitude	Salinity (psu)
AB: Aransas Bay	28°01'51.7"N	96°59'53.2"W	21.8 (± 1.8)
LC: Lake Calcasieu	29°50'58.02"N	93°17'1.32"W	18.2 (± 5.0)
SL: Sister Lake	29°14'30.77"N,	89°29'52.45"W	10.8 (± 5.5)
VB: Vermilion Bay	29°28'52.32"N	90°54'30.24"W	3.4 (± 2.6)

Fig. 1 Map indicating oyster collection sites across the Gulf of Mexico. Louisiana oysters came from one of the three sites shown here. Below the map, the table displays coordinates and mean salinity (±SE) for each site.

Single-pair crosses were performed in two different years: Four Texas and two Louisiana crosses were performed in 2018 and four Louisiana crosses were performed in 2019 (Table 1). For all crosses, oysters were strip spawned, sperm was passed through a 13-μm filter, and eggs were passed through a 35-μm filter to remove debris. Fertilization occurred at 28°C and incoming seawater salinity: 30 psu for 2018 crosses and 15 psu for 2019 crosses.

Following 12-h post-fertilization (hpf), we subjected larvae from each cross to a low salinity selection experiment. Within 12–24 hpf, larvae begin to develop their shell (Wallace et al. 2008), with reduced survival previously measured under low salinity exposure at this sensitive life stage (Scharping et al. 2019). Prior to selection, we took a sample of approximately 600,000 larvae, which were immediately flash-frozen for pre-selection genetic analyses. We subjected the remaining larvae to a low salinity of either 7, 4, or 3 psu. We chose the salinity for each selection experiment based on preliminary tests of what salinity would provide at least 50% larval knock-down in each set of crosses. All crosses performed in 2018 (four Texas crosses and two Louisiana crosses) were exposed to 7 psu (Table 1). In 2019, we chose slightly lower salinities (3 and 4 psu) to increase the strength of selection while also trying to prevent 100% mortality (Table 1). Each cross was exposed to the low salinity stress separately at a concentration of ~250 larvae/mL. Following 12 h of low salinity exposure, we carefully collected the swimming larvae by pouring off the top 90% water to ensure larvae that had died (or had been knocked-down) were not disturbed in the bottom of the beaker. We concentrated the larvae that were swimming in the top part of the beaker and immediately flash-froze them for post-selection genetic analyses. We confirmed a selection event had occurred by assessing mortality in the non-swimming portion of the beaker using a neutral red assay to estimate the % mortality. Finally, we calculated the strength of selection from low salinity, genetic load, and laboratory exposure, by estimating the total number of surviving larvae collected in the swimming portion of the beaker.

For one of the Louisiana crosses (LA cross 6), we also performed a control experiment, where larvae were kept at their ambient spawning salinity for 12 h. After the 12 h of exposure, we sampled surviving larvae as described

Table 1 For each cross, the low salinity (psu) exposure is displayed along with metrics for the strength of selection (% swimming and total number of larvae used for post-selection genetic analyses) and the total number of genes under selection

Cross	Salinity (psu)	Exposure year	Swimming (%)	Swimming (# larvae)	# genes under selection
LA 1	7	2018	1.27	7654	3
LA 2	7	2018	0.56	3383	1
LA 3	3	2019	1	5500	30
LA 4	4	2019	2	12,000	2
LA 5	4	2019	2	15,000	0
LA 6	4	2019	4	20,000	0
LA 6 NS	15	2019	10	132,000	0
TX 1	7	2018	0.38	2337	4
TX 2	7	2018	20	123,600	0
TX 3	7	2018	33	199,800	4
TX 4	7	2018	67	406,008	13
LA average	**7/4/3**	**2018/2019**	**1.81**	**5518**	**49 unique**
TX average	**7**	**2018**	**30.1**	**108,579**	**20 unique**

above and subjected this sample to the same analyses as described below.

Exome capture design and pool-sequencing

We created probes for exome capture of 152 genes that have previously been identified in four different studies for low salinity tolerance in oysters. We identified 47 genes that show high levels of differential gene expression in Louisiana populations of *C. virginica* when exposed to low and high salinity (Jones et al. 2019). We identified 26 genes that show high F_{ST} between low and high salinity sites in Louisiana populations of *C. virginica* (Johnson and Kelly 2019). We also identified genes that are associated with low salinity tolerance in a closely related species, *C. gigas*. This included 33 genes with high levels of polymorphism associated with salinity adaptation between populations of *C. gigas* (She et al. 2018). Another 30 genes were identified from *C. gigas* that show changes in free amino acid metabolism pathways (such as taurine, glycine, proline, alanine, etc.) (Meng et al. 2013). For genes identified in *C. gigas*, we used BLAST to identify orthologs in the *C. virginica* genome. There were 27 genes that fell into two or more source categories (Fig. S1). We also haphazardly identified 42 genes to ensure equal representation of sequencing across the genome (referred to as "other genes"). The 152 genes were grouped into 12 functional categories: chemical defense, immune response, ion and water balance, elongation factors, free amino acid metabolism, fatty acid hydrolysis, ubiquitin-related, proteolysis, reactive oxygen species (ROS), RNA polymerase, free amino acid rich proteins, or uncharacterized. Probes were designed to start 2000 bp upstream of the gene and extend for 8000 bp. Detailed information on each gene (including sequenced start and end positions, function, and source category) can be found in Table S1.

One sequencing library was made from each parent and each pooled larval sample pre- and post-selection and one additional cross with no selection for a total of 41 libraries. DNA was extracted using an OMEGA E.Z.N.A (Norcross, GA, USA). Tissue DNA extraction and quantity was assessed using a NanoDrop spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA). We constructed whole genome libraries from sonicated DNA using the NEBNext Ultra DNA Library Prep Kit for Illumina (Illumina, San Diego, CA, USA). Concentration and fragment size distributions for each library were assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA). Each library received an individual barcode adapter using the Multiplex Oligos for Illumina. We pooled four to five libraries prior to exome capture based on similar fragment size distributions. Probes were hybridized overnight using the myBaits® Target capture kits (Arbor Biosciences, Ann Arbor, MI, USA). Samples collected in 2018 were sequenced on half a lane of an Illumina HiSeq 3000 with a goal coverage of 100x (assuming a 40% capture efficiency). Single-end (1 × 150 bp) sequencing was performed at Iowa State University's DNA Facility (Ames, IA, USA). Samples collected in 2019 were sequenced on half a lane of an Illumina HiSeq X

with a goal coverage of 100x. Paired-end (2 × 150 bp) sequencing was performed at NovoGene (Beijing, China).

Detecting changes in allele frequencies

Low-quality bases (Phred score < 20) and adapters were removed using FastQC v0.11.5 (Andrews 2010). Reads were mapped to the *C. virginica* genome (GenBank accession number: GCF_002022765.2) using Bowtie2 (Langmead and Slazberg 2013). We then followed the pipeline outlined by Popoolation2 (Kofler et al. 2011) to analyze pool-sequencing data. Ambiguously mapped reads were removed using samtools view and sort (Li et al. 2009). A synchronized file containing single nucleotide polymorphism (SNP) frequencies for each sample was created using the samtools mpileup option (with a minimum quality Phred score of 20) and the perl script mpileup2sync.pl through Popoolation2. We visualized genome-wide relationships among sample allele frequencies using a principal coordinate analysis (PCoA) and the Adonis function in the R package vegan (Oksanen 2015). Manhattan distances were computed from allele frequencies for each larval sample pre- and post-selection in the PCoA.

Next, we performed Fisher's exact test to identify strong changes in allele frequencies pre- and post-selection for each cross using a required coverage of 20–200x for each SNP. We then used a false discovery rate correction for individual SNP *P*-values and a Bonferroni significance threshold. We were interested in following the fate of alleles that are present in the heterozygous state in one of the parents. Since we performed single-pair crosses, minor alleles are expected to have a starting frequency of ∼25% in the offspring. Therefore, we filtered SNPs by requiring a starting frequency (pre-selection) between 15 and 35%. This conservative window omits SNPs that are of lower frequency due to sequencing error. We also required SNPs to increase in frequency post-selection to identify SNPs that show a positive response to low salinity selection. (The total number of SNPs for each cross that passed these thresholds are reported in Table S2.) The mean starting frequency after filtering was 24.2% (±5.7% standard deviation; Table S3). To account for large linkage blocks because of our single-pair cross design, we required at least three significant SNPs within a gene to categorize a gene as under selection for low salinity performance. To determine if the number of shared genes under selection among crosses is greater than expected by chance, we performed a hypergeometric test in R v.4.0.3.

Annotation of genes under selection

We determined if genes under selection were enriched in a particular functional or source category (e.g., high gene expression, high F_{ST}, etc.) by performing a Chi-squared test in R. To determine if SNPs were more likely to be upstream of the gene (and therefore potentially involved in regulatory changes), we first identified the location of genetic variation in the pre-selection larval samples (SNPs with a starting frequency between 15 and 35%). We then performed a Chi-squared test in R to determine whether SNPs post-selection were more likely to be found upstream, within, or downstream of the genes based on SNP distributions pre-selection.

Results

Validation of selection experiment

We confirmed that a lethal selection event had occurred (from both low salinity exposure and high genetic load) by estimating the % survival of larvae in the water column of the beaker. We observed survival rates between <1 and 67%, confirming that selection was occurring in each cross. (Table 1). The Texas crosses had a mean survival rate of 30%, while the Louisiana crosses had a mean survival rate of 1.81%. High survival rates in the Texas crosses may be explained by the comparatively weak selection event that was imposed; the Texas crosses were exposed to a salinity of 7 psu, which was not as extreme as the low salinity exposure experienced by some of the Louisiana crosses. However, the Louisiana crosses were exposed to a range of low salinities (3–7 psu) but had similar survival rates across all salinities. Alternatively, differential mortality could be explained by higher genetic load in oysters from Louisiana, or potentially from "bucket effects" since crosses were not replicated.

Genetic change post-selection

The mean number of raw reads per library was 6,253,219 with a mean mapping rate of 55.5% and mean coverage of 75.9x. (Results are reported for parent and larval samples in Table S4; however, only larval samples were included in the final analyses). A total of 61,974 SNPs among larval samples were genotyped and used to estimate genetic diversity in the PCoA (Fig. 2A and B). Overall, larval oysters showed significant clustering by population (Adonis $P_{population} < 0.01$; Fig. 2) as well experimental year (Adonis $P_{year} < 0.01$). We note that only crosses from Louisiana were performed in 2019, thus potentially confounding population differences with experimental year. To mitigate further batch effects, we compared the distances within crosses pre- and post-selection to determine treatment effects. We

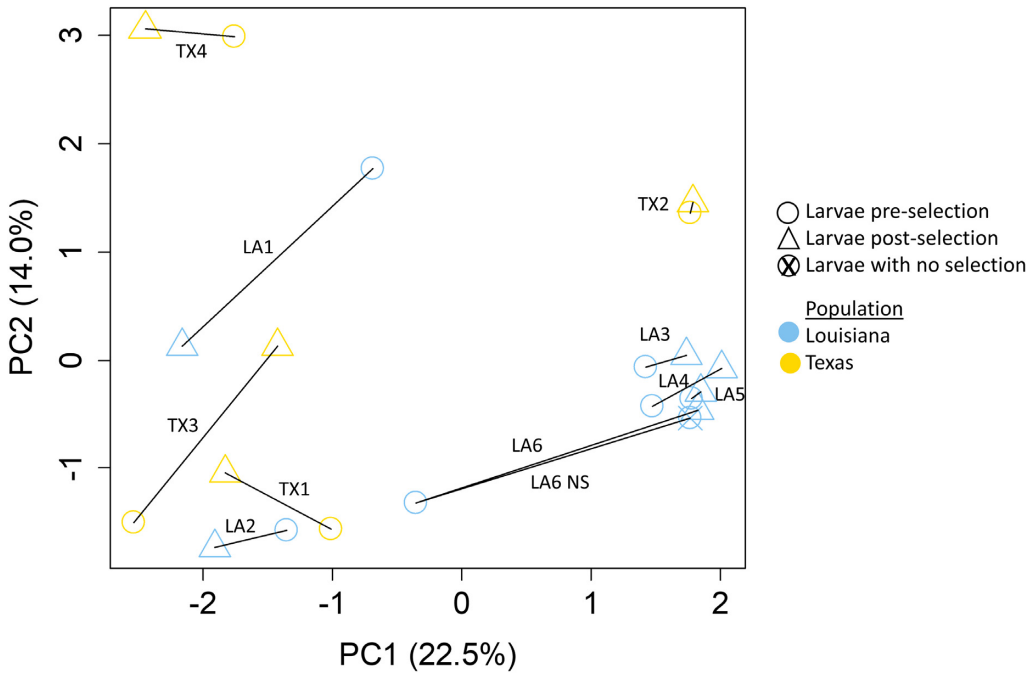


Fig. 2 PCoA of allele frequencies for larval samples pre- and post-selection for low salinity and genetic load and one control with no low salinity selection (i.e. genetic load selection only). Oysters originating from Louisiana are yellow and oysters originating from Texas are blue. Crosses pre- and post-selection are connected with a line. Circles are larvae pre-selection, triangles are larvae post-selection, and the circle with an X are larvae with no low salinity selection.

found that overall distances on the PCoA between larvae pre-and post-selection were not correlated with the number of genes under positive selection in that cross ($F_{1,9} = 0.45$, $P = 0.52$), nor were distances correlated with the strength of low salinity exposure (i.e., 3, 4, or 7 psu; $F_{1,9} = 1.04$, $P = 0.34$), population origin ($F_{1,9} = 0.17$, $P = 0.69$), or mortality ($F_{1,9} = 0.25$, $P = 0.63$). This suggests that genetic load may be the primary driver of allele frequency changes as larvae purge deleterious alleles (Launey and Hedgecock 2001; Li and Guo 2004). Unique deleterious alleles present in each parent or mutations acquired during meiosis could result in each cross responding in a different direction in PCoA space. To account for this variation, we filtered SNPs that increased in frequency after salinity exposure to identify positive selection acting on the variation responsible for salinity performance.

For the Texas crosses, we observed significant increases in allele frequencies in three of the four crosses (TX crosses 1, 3, and 4; Fig. 3), with a corresponding mean increase in frequency of 0.14, 0.16, and 0.19 for each cross, respectively, for significant SNPs (Fig. S2E–G). However, we saw no variation segregating under selection in TX cross 2 (Fig. S3), suggesting that not all individuals in the population harbored variation for larval performance that can be acted upon by selection. For the Louisiana crosses, four of six crosses had alleles segregating under positive selection (LA crosses 1–4; Fig. 3), with a corresponding mean increase in frequency of 0.17, 0.20, 0.17, and 0.15 for each cross, respectively, for significant SNPs (Fig. S2A–D). One of these crosses (LA cross 3) had 30 genes that were under selection, suggesting that there is ample variation for larval performance in this cross. For LA cross 6, we also performed a control experiment, whereby larvae were kept at their ambient spawning salinity for 12 h. In this cross, we expect the allele frequency changes to be proportional to the amount of genetic load present. We observed no significant increases in allele frequencies for LA cross 6 exposed to low salinity nor for the control experiment (Fig. S3), and both crosses experienced the same change in PCoA space. Together, these results suggest that LA cross 6 did not have the genetic variation needed for low salinity tolerance.

For the crosses that did have significant increases in allele frequencies, there were 11 genes that were under selection in multiple crosses (Table 2). The number of shared genes among crosses was greater than expected by chance for TX1, TX3, TX4, and LA3 (hypergeometric test: $P < 0.0001$; Table S5), providing evidence for the importance of these genes for low salinity tolerance.

Finally, we designed probes to sequence ~2000 bp upstream of each gene body to determine whether variation was more likely to be upstream rather than within the gene body. An excess of variation upstream

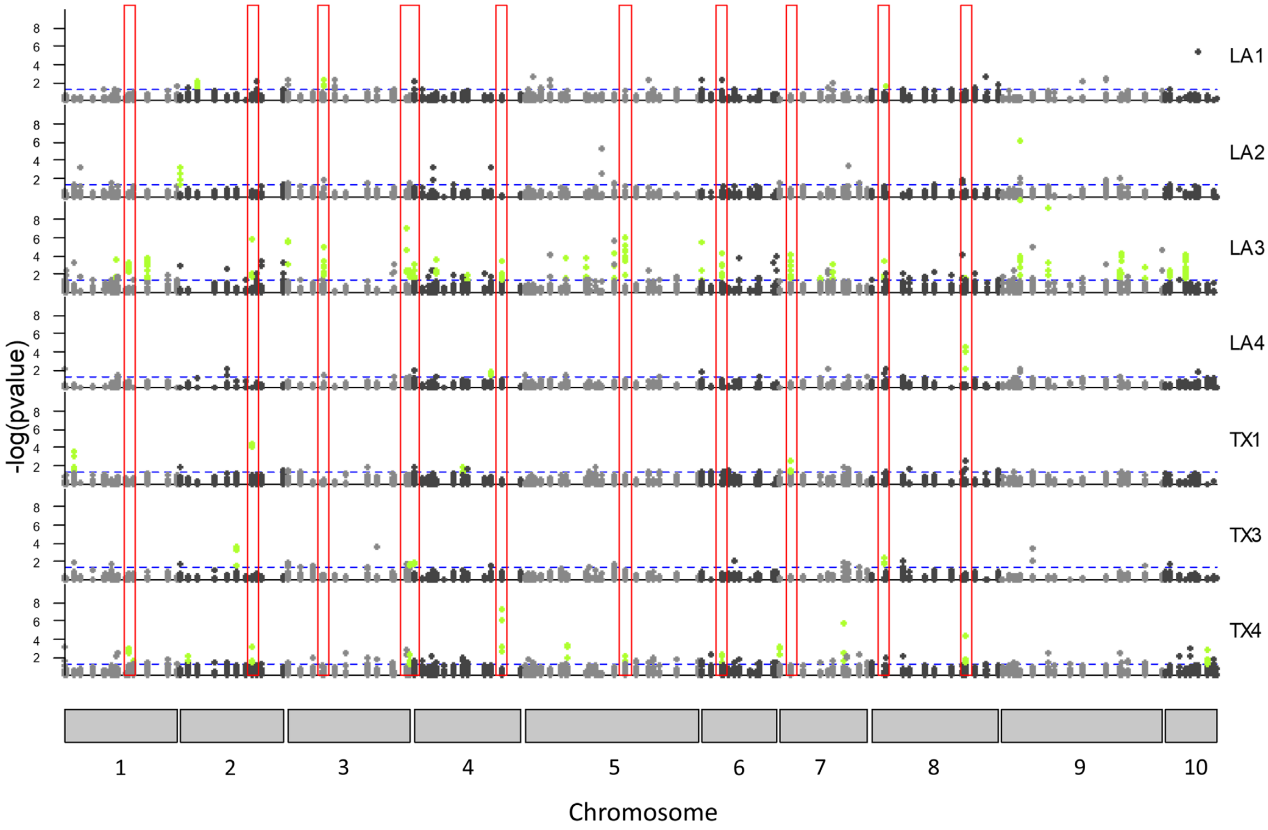


Fig. 3 Manhattan plots for crosses showing SNPs that had a consistent increase in allele frequency post-selection for low salinity tolerance. *P*-values are displayed as –log from Fisher's exact test. The blue horizontal line represents our significance threshold after Bonferroni correction. SNPs colored green pass our threshold for considering the gene to be under selection (three significant SNPs per gene). Red boxes highlight the SNPs for each gene that is under selection in more than one cross.

Table 2 Location and identity for genes under selection in more than one cross

Chromosome	Gene	Cross	Functional grouping	Source
5	22430406	LA3, TX4	Immune system	High F_{ST}
6	22430888	LA3, TX4	Chemical defense	She (2018)
7	22436855	LA3, TX1	Ubiquitin	Other
8	22446194	LA3, TX3	Immune system	Other
8	22447067	LA3, LA4, TX4	Chemical defense	She (2018)
1	22455865	LA3, TX4	Ion/water balance	High gene expression
2	22458334	LA3, TX1, TX4	ROS-related	She (2018)
3	22464985	LA3, TX1, TX4	Ion/water balance	She (2018) and Meng (2013)
3	22467005	LA1, LA3	Uncharacterized	High F_{ST}
4	22473715	LA3, TX3	AA protein	High gene expression
4	22477495	LA3, TX4	Fatty acid hydrolysis	High F_{ST}

of the gene body would suggest that regulatory (or plastic) changes may be more important than structural changes for low salinity and high genetic load selection. In only one cross, we observed an increase in SNPs found upstream post-selection (LA cross 4; $P < 0.001$; Table S6). Another cross was more likely to have SNPs increase in frequency within the gene body post-selection (LA cross 1; $P = 0.026$; Table S6). For all other crosses with genes under selection, SNPs were not more likely to be upstream than within the gene body ($P > 0.05$ for all other crosses; Table S6). Thus, we were unable to resolve the relative importance of regulatory

(i.e., plastic) versus structural changes for adaptation during early life stages.

Annotation of genes under selection

Salinity tolerance is most likely a polygenic trait with many mechanisms of resilience; however, we found that genes under selection were not more likely to be enriched in a particular functional or source category ($P > 0.05$ for all crosses; Table S6). This may be an artifact of our experimental design, where we chose genes with known functions in salinity tolerance. In addition, we only sequenced a small portion of the genome, and large linkage blocks are likely preventing us from identifying the true targets of selection.

Discussion

As salinity conditions in the Gulf of Mexico are predicted to decline due to anthropogenic activities, productive oyster breeding grounds are at risk. One avenue oysters may take to respond to this rapid environmental change is evolutionary adaptation. In this study, we have evaluated the capacity of *C. virginica* to respond to combined low salinity selection events and high genetic load using experimental evolution over a single generation. We found that standing genetic variation exists for performance under ecologically relevant salinity regimes and high genetic load, suggesting that selection during the larval phase could result in substantial changes in allele frequencies present in adult populations.

Presence of standing genetic variation

We observed increases in allele frequencies in multiple crosses from Texas and Louisiana populations. In addition, multiple chromosomes had genes under selection in each cross, suggesting that low salinity performance is polygenic, as has been demonstrated in other species adapting to environmental stressors (Rodríguez-Trelles et al. 2013; Bergland et al. 2014; Bitter et al. 2019; Brennan et al. 2019; Griffiths et al. 2020). The number of shared genes under positive selection among crosses was greater than expected by chance (Table S5). Deleterious alleles segregating in a population under mutation–selection balance are expected to be rare, with an equilibrium frequency equal to the product of the selection strength in heterozygotes and the mutation rate ($q = \mu hs$). The probability of sampling the same rare allele twice in a single experiment is proportional to the square of its frequency, which is vanishingly small in the case of rare alleles. By contrast, environmental heterogeneity and temporally varying selection are expected to maintain ecologically relevant alleles under intermediate frequencies. Therefore, we argue that the instances in which we have observed selection on the same gene in two or more crosses are more likely to be instances of selection on salinity tolerance alleles, rather than being a consequence of genetic load alone. Since we expect large linkage blocks to be present in our experiment, it is also possible that these genes are within linkage blocks that contain the true target of low salinity tolerance.

Our unique experimental design also allowed us to identify genotypes within a population where genetic variation for salinity tolerance resides. Many experimental evolution studies utilize large factorial breeding designs to achieve recombination of many genotypes. However, it is unclear if the changes in allele frequencies observed are the result of dominance from a single, rare, genotype that has the highest fitness or if multiple genotypes contribute to resilience. In our study, we demonstrate variation in survival among families for low salinity performance, suggesting that some genotypes are more resilient than others. Additionally, not all crosses had genetic variation that segregated under low salinity tolerance stress, suggesting that some individuals within the population lacked heterozygous loci involved in low salinity performance for our chosen genes.

Maintenance of genetic variation in wild populations

These results provide insight into how genetic variation may be maintained in wild populations. Oysters from Louisiana estuaries are naturally exposed to lower salinity conditions than oysters from Aransas Bay, TX; thus, we might expect oysters to be locally adapted to their salinity regimes. However, oysters from Texas had lower mortality than Louisiana oysters under low salinity, suggesting that oysters distributed across the Gulf of Mexico may not be locally adapted to salinity. Instead, mortality may have depended on the amount of genetic load present in each cross. A recent study also found little evidence for local adaptation to salinity among the same populations of *C. virginica* as used in this study (Marshall et al. 2021). Another pattern of local adaptation manifested at the genetic level would be reduced genetic variation for low salinity performance in Louisiana oysters. Recurring low salinity selective sweeps are expected to reduce genetic diversity after multiple generations of selection, and salinity tolerance alleles would be swept to higher frequencies (Przeworski 2002; Savolainen et al. 2013). Instead, we observed multiple Louisiana crosses with genetic variation for low salinity performance and genetic load. This variation may be maintained by a few possible

mechanisms. First, high gene flow among populations may promote balanced polymorphism for salinity performance. In this scenario, the migration–selection balance in the wild is not strong enough to remove unfit alleles from the environment (Savolainen et al. 2013), or temporally fluctuating stressors may maintain all genotypes in a population (Pedersen et al. 2000). Second, low salinity tolerance may have negative consequences and trade-offs might exist (Mitchell-Olds et al. 2007). Thus, high salinity genotypes in the population may still have a fitness advantage that was not measured in this study. Third, our results may be indicative of the sweepstakes hypothesis, which attributes reproductive success to the likelihood of gametes and offspring encountering conditions conducive to development (Hedgecock and Pudovkin 2011), or, conversely, from a genotype-by-environment mismatch (Marshall et al. 2010). These hypotheses explain patterns of low genetic diversity observed within populations of *C. virginica* (Johnson and Kelly 2019), but high genetic diversity among cohorts. Our results lend support to the hypothesis that selection for ecologically relevant salinities during the larval phase acts as a filter on genotypes found in adult populations.

High genetic load and purifying selection

High mortality has been observed in the larval stage (Rumrill 1990), but distorted Mendelian segregation ratios have only been observed at the juvenile stage (2 months–1 year old) (Launey and Hedgecock 2001; Li and Guo 2004). Our results suggest that the purging of deleterious alleles may occur as early as 12–24 hpf in *C. virginica*; we observed large changes in PCoA space in our control experiment. Most of the natural variation observed was likely driven by the presence of rare deleterious mutations in our crosses. This presents a challenge to identifying the causative SNPs under salinity tolerance selection, since many linkage blocks may contain loci under strong purifying selection, making it difficult to detect alleles under positive selection for salinity tolerance. Therefore, we focused on alleles that increased in frequency, and we identified a putative set of genes that may be under selection for low salinity performance. However, high linkage disequilibrium (LD) may prevent us from being able to capture the full catalog of alleles important for low salinity survival.

Mechanisms of response to low salinity

We sequenced genes that change their expression patterns in response to salinity or have unique polymorphisms among populations distributed across salinity gradients. We demonstrated that these genes may be important for adapting to future declines in salinity. Loci that were under selection in multiple crosses suggest that salinity performance has many mechanisms of resilience, such as water and ion balance, immune system, and chemical defenses (Table 2). However, we note that these may not necessarily be the target of selection and that these loci could also be linked to the true loci under salinity selection or loci with high genetic load. Future work using whole genome sequencing may help further elucidate the true loci under selection.

Conclusion and future directions

As estuaries within the Gulf of Mexico undergo drastic changes in salinity patterns, understanding how oyster populations will respond, and identifying resilient genotypes within populations, will inform resource management and hatchery practices. Our unique experimental design utilized single-pair crosses, which may be more informative for hatchery managers when selecting broodstock that are resilient to ecologically relevant stressors. We provide a putative set of alleles that may be responding to low salinity selection and high genetic load in both Texas and Louisiana populations, which would suggest *C. virginica* has the potential to respond and adapt to future salinity changes in the wild. However, the lack of experimental controls for all but one cross in our experiment has prevented us from concretely identifying the targets of salinity selection. To better measure a genotype-by-environment interaction that would drive divergent responses to salinity selection, control experiments for each family should be performed in parallel with each low salinity exposure. This approach would allow us to fully disentangle loci responding to low salinity selection and those that fall out due to high genetic load. Furthermore, larvae that survive the salinity challenge could be followed to the juvenile and adult stages to determine whether the observed changes in allele frequencies from this experiment influence long-term survival in a manner that could contribute to rapid adaptation to climate change.

Author contributions

M.W.K. conceived the study. J.S.G., K.M.J., and M.W.K. performed crosses and experiment. J.S.G. prepared libraries, performed analyses, and wrote the manuscript. All authors approved the final version of the manuscript.

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Supplementary data

Supplementary data available at *ICB* online.

Data availability

Data and scripts are deposited at https://github.com/JoannaGriffiths/Evolutionary_Change_in_Eastern_Oyster.

Raw sequencing data are deposited in the National Center for Biotechnology Information's Short Reads Archive (GenBank accession number: PRJNA699020).

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