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Multiple drivers of interannual oyster settlement and recruitment in the lower Chesapeake Bay

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ABSTRACT: Despite global investment in shellfish restoration activities, relatively little attention has been given to predicting optimal restoration sites and testing these expectations. We used a coupled biological-physical connectivity model as a guide to plant two distinct hatchery-spawned strains of the eastern oyster, *Crassostrea virginica*, in the Lafayette River, Virginia during the summer of 2013 at two locations corresponding to virtual spawning locations within the connectivity model. We utilized single nucleotide polymorphism markers to test the model predictions by genotyping oyster recruitment the year after planting and examining interannual recruitment variability for two successive years. None of our experimental oyster genotypes were detected; however, we did observe a genetic influence from an oyster strain used previously for restoration. Differences in environmental conditions between the two years of monitored recruitment likely affected larval dispersal and survival contributing to observed interannual differences in the demographics of newly recruiting cohorts. Oyster recruits from 2013 were genetically more similar to resident adults sampled in the Lafayette River, while the 2014 recruits exhibited genotypic frequencies more similar to adults from surrounding rivers. The winds during the spawning seasons differed between years providing conditions for retention in 2013 and mixing of water masses in 2014. We recommend that the monitoring of restoration activities should consider relevant environmental conditions and observe multiple years of recruitment to assess the genetic impacts of restoration plantings and variable reproductive success.

KEY WORDS: citizen science; connectivity; *Crassostrea virginica*; restoration; seascape genetics; single nucleotide polymorphism

INTRODUCTION

Traditionally, population genetic studies of larval and juvenile dispersal have focused primarily on the correlation between genetic and geographic distances to understand population connectivity. More recently, contemporary landscape genetics methods used in terrestrial environments have considered environmental data in addition to geographic distances to explain regional population structure; however, the marine environment presents unique challenges (Selkoe et al. 2016). For example, ocean surface currents forced by winds and tidal cycles are two important factors impacting the distribution and survival of estuarine organisms. As a result, the physical dynamics of estuaries are vital to understanding resident organisms, and hydrodynamic models that incorporate biological components are valuable for formulating population connectivity hypotheses. Restoration projects located in estuaries could benefit from the combination of physical modeling and population genetic analyses to better predict optimal restoration sites and track the reproductive influence of restoration introduced animals.

Molecular markers have been used for tracking the efficacy of restoration projects by monitoring the genetic contribution of planted animals to newly recruiting individuals with varying success. In 2002, the Virginia Marine Resources Commission (VMRC) working with Virginia Institute of Marine Science (VIMS) researchers planted a selectively bred strain of oyster in the Great Wicomico River, Virginia with the goal of using nuclear microsatellite markers to track the progress of restoration efforts (Hare et al. 2006, Carlsson et al. 2008). Hare et al. (2006) identified a single oyster spat (of 1579) as a full offspring of the restoration planted oysters and 153 (9.7%) as F1 hybrids. Milbury et al. (2004) successfully used mitochondrial 16S ribosomal DNA sequences to detect genotypes specific to oysters planted for restoration in Delaware Bay. Although the percentage of recovered spat, spat defined as post-larval oysters less than 6 months old, assigned to oysters introduced during restoration efforts was low (~0.08%), the results confirmed that introduced oysters were contributing to reproductive output. Wilbur et al. (2005) determined that a small percentage of bay scallop (*Argopecten irradians*) recruits (~0.03%) were genetically related to restoration planted animals on the west coast of Florida based on two randomly amplified mitochondrial DNA (mtDNA) fragments. While these previous tracking studies had lower returns than expected *a priori*, they did demonstrate the utility of molecular markers for monitoring the reproductive contribution of restoration planted organisms.

The eastern oyster, *Crassostrea virginica*, has a continuous distribution along the eastern coast of North America ranging from the Yucatan Peninsula in the south to Nova Scotia in the north (Buroker 1983). The

Chesapeake Bay is an important habitat for *C. virginica* and this region has a history of fishery exploitation contributing to population declines of oysters and reduction in the number of oyster reefs (Jackson et al. 2001). The ecosystem value of oysters has been estimated to range from $5,500 to $99,000 per hectare per year (Grabowski et al. 2012), and government agencies and non-governmental organizations have invested significant resources in oyster restoration within the Chesapeake Bay (USACE 2012). Restoration projects that plant live animals into the ecosystem assume that the restoration oysters will supplement the wild populations and contribute to future reproduction. Despite the emphasis on restoring oyster reefs in the Chesapeake Bay, there has been relatively little attention given to assessing the direct reproductive contribution of oysters planted during restoration activities.

Our study was a collaboration that has its origins in *C. virginica* restoration efforts managed by the Chesapeake Bay Foundation (CBF). Since 1999, CBF has constructed oyster reefs in the Lafayette River, Virginia and planted oysters on many of these artificial reefs. To maximize the impact of the restoration efforts, the CBF contracted a modeling group at VIMS to develop a biological-physical connectivity model (referred to as connectivity model), which included oyster larvae behavior, to predict locations that would have the greatest larval export potential and locations that would have the greatest larval settlement in the Lafayette River (Sisson and Shen 2012). The CBF used the predictions of the connectivity model as a guide to plant two hatchery-spawned strains in the Lafayette River during the summer of 2013 at two locations corresponding to virtual spawning locations within the model. We developed a panel of single nucleotide polymorphism (SNP) markers and used them to determine whether there was evidence that genetically characterized, planted oysters were reproducing and contributing to annual recruitment within the Lafayette River. Moreover, we compared the settlement and recruitment of oysters between two successive years in the Lafayette to better understand differences on interannual timescales. We also examined two years of oyster settlement data in the Lafayette River to make a qualitative comparison to the predictions produced by the biological-physical model created by Sisson and Shen (2012).

METHODS

EXPERIMENTAL OYSTER PLANTINGS

CBF introduced two hatchery-spawned oyster strains into the Lafayette River during the summer of 2013 to experimentally track restoration-associated recruitment within the Lafayette. Adult oysters ($n_{females} = 35$, $n_{males} = 12$) were collected from Cod Harbor, Tangier Island, Virginia (Figure 1a) by VMRC and strip-spawned at Oyster Seed Holdings, LLC, Goodwin Island, Virginia in July 2013. About 1.8 million hatchery-spawned Tangier Island oysters

were deployed as spat attached to cleaned oyster shell at the Granby Street Bridge reef (Figure 1b) in August 2013. Oysters from Tangier were used for this study because Tangier Island is geographically distant from the Lafayette River (>100 km), the prominent larval dispersal patterns do not favor direct connectivity between the two systems (North et al. 2008), and previous research found a pattern of isolation by distance for *Crassostrea virginica* in the Chesapeake Bay (Rose et al. 2006). These lines of evidence suggested that oysters from Tangier and the Lafayette would be genetically distinct from each other. Additionally, about 114,000 Northeast High-Survival line (NEH®) cultchless oysters, which are individual oysters not attached to substrate, were deployed at the Larchmont reef (Figure 1b) in August 2013. The NEH® oysters were spawned at Rutgers Haskin Shellfish Hatchery in Port Norris, New Jersey during November 2012 and were donated by CBF from a decommissioned oyster lease located at Sarah's Creek, Virginia. The NEH® oysters were used because they are a selectively-bred strain that are reproductively capable and was known to be sufficiently different from wild oysters in the lower Chesapeake Bay (defined here as the James, Elizabeth and Lafayette Rivers) to be used for recruitment tracking using genetic methods. The experimental oysters were planted on separate reefs chosen to coincide with virtual oyster larvae release locations within the Sisson and Shen (2012) connectivity model. The experimental planting locations were selected as a trade-off between model predictions, existing restoration reef locations, and locations with reasonable survival probability due to habitat characteristics. The Granby reef was predicted to have the second highest larval production potential after a location upriver (Sisson and Shen 2012). The upriver location with highest larval production potential is muddy and subject to environmental extremes not suitable for larval survival. The Larchmont reef was already the site of an extensive artificial reef with high densities of healthy oysters.

OYSTER SAMPLING

Adult oysters were sampled from multiple locations in the Chesapeake Bay to serve as reference groups to compare against spat sampled in the Lafayette River (Figure 1a). Oysters were acquired from the Elizabeth, James, and Great Wicomico Rivers by the VMRC vessel *J. B. Baylor* with hydraulic patent tong (methods detailed by Southworth and Mann 2015). Sampling was conducted during annual oyster surveys in the autumns of 2013 and 2014 from randomly selected locations in the three rivers; however, the Great Wicomico was only sampled in 2014 by dredge. Rappahannock River samples were obtained during the summer of 2015 from an annual naïve oyster disease survey conducted by researchers at VIMS. In the Elizabeth River, Hospital Point and the Western Branch reefs were sampled. Cruiser's Rock, Wreck Shoals, and Upper Deep-Water Shoal reefs were sampled in the James

River. Colley Avenue Bridge, the seawall on Mayflower Road, and Granby Street Bridge were sampled in the Lafayette River. In addition, subsamples of the NEH® oysters and Tangier Island offspring, which were planted in the Lafayette River as our experimental oysters, were collected for genotyping. For this paper, samples refer to groups of individuals collected in the same river system as reference and groups of individual spat obtained from spat-collectors in same year. Sample abbreviations are as follows: Lafayette River adult reference (LR), Lafayette River 2013 spat (LR13), Lafayette River 2014 spat (LR14), Elizabeth River reference (ER), James River reference (JR), Rappahannock River reference (RR), Great Wicomico River reference (GR), Tangier Island offspring (TI), and Northeast High-Survival Line (NEH®).

The CBF's citizen-science spat-collector program was used to quantify the magnitude of oyster recruitment into the Lafayette River and to obtain spat samples for genotyping. The spat collectors were placed on the docks of volunteers within the Lafayette River from May through October of 2013 and 2014. Collectors were positioned approximately 15 centimeters off the bottom and agitated weekly to remove sediment. Spat from retrieved spat-collectors were returned to the lab, and proportionate sampling was used to obtain a representative sample of the spat distribution from the river.

Shell-string surveys were used to quantify larval supply and settlement timing within the Lafayette River. This survey was designed to capture late-stage larvae recently attached to substrate. The surveys were conducted at the docks of four volunteers in the Lafayette River from May through October in 2013 and 2014. One location was upriver, two were mid-river, and one was near the mouth of the river (Figure 1b). Shell-strings consisted of ten cleaned oyster shells with a hole drilled in the center and hung on 12.5-gauge wire. The shell-strings were suspended from docks about 15 cm off the bottom. Every other week the old shells were removed for sampling and replaced with new shells. A dissection microscope was used to enumerate larvae that settled on the oyster shells.

OYSTER GENOTYPING

Gill, mantle, and adductor-muscle tissue were sampled via sterile technique for DNA extraction and were preserved in 95% ethanol. Genomic DNA was isolated from tissue samples using either the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) or the Genomic DNA – Tissue MicroPrep Kit (Zymo Research, Irvine, CA) using the manufacturers' protocols. Concentration and purity of extracted DNA was quantified on either a NanoDrop 2000 or Qubit spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and only samples with concentrations greater than 5.0 ng/µl were used for genotyping.

The SNP markers used in this study were designed based on published (Zhang and Guo 2010) and unpublished loci originally developed from expressed sequence tags to assess disease resistance in hatchery-selected strains of *C. virginica*. A total of 95 loci were converted to SNPtype assays (Fluidigm, San Francisco, CA) using the D3™ Assay Design software (Fluidigm) and screened for reproducibility. The final assay panel consisted of 48 SNP loci, and details of panel development can be found in Online Resource 1. Oysters were genotyped on a 48 x 48 Dynamic Array IFC (Fluidigm) using the manufacturer's recommended protocol. Briefly, pre-amplification PCR was done first using specific target amplification (STA) primers for each SNP locus to standardize the quantity of starting DNA template for each sample before input into the SNPtype assay reaction (see Online Resource 2 for primer sequences). The STA products were diluted 1:100 in DNA Suspension Buffer (Teknova, Inc., Hollister, CA). SNPtype assay reactions using the ASP primers (see Online Resource 2 for primer sequences) were performed on a Fluidigm IFC-1 cycler using the manufacturer's recommended protocol and the diluted STA products as template on a 48 x 48 Dynamic Array IFC, which genotypes 47 individuals plus one blank control and 48 SNP loci simultaneously. Resulting fluorescence signals were recorded on a BioMark HD and data were processed using the SNP GENOTYPING ANALYSIS 4.1.2 software **(**Fluidigm, San Francisco, CA**)** using a k-means clustering algorithm. The confidence level for clustering was set at 65%, as recommended by the manufacturer, and cluster membership was verified by eye. Samples that had relative fluorescence values below 0.3 were invalidated and the data were re-clustered. Ambiguous genotypes, defined as individual fluorescence values that were far from other genotype clusters or half way between two clusters, were excluded to minimize genotyping errors. During marker development, 141 individuals were run twice, and consistent results were obtained between replicate runs. There was variance in the fluorescence values for the individuals run twice, but the genotype of each individual was consistent between the duplicate runs. Of the 48 loci chosen for our SNP panel, one locus was chosen as a duplicate to serve as an analytical control to assess reproducibility. The duplicate locus was removed from the data before downstream analyses were performed. Due to reproducibility issues only 41 loci were used in the statistical analyses (see Online Resource 1)

STATISTICAL ANALYSIS

Population genetic summary statistics were calculated for the data within and between sampled groups. Oyster genotypes were checked for conformance to the expectations of Hardy-Weinberg Equilibrium (HWE) using exact tests implemented in PEGAS 0.9 (Paradis, 2010) in R 3.4.0 (R Core Team, 2017). Individual locus F-statistics

were calculated as described by Weir and Cockerham (1984) in HIERFSTAT 0.01-14 (Goudet, 2014) in R. In addition, minor allele frequencies were calculated for each SNP locus to assess rare alleles in a sampled group. Tests for genotypic linkage equilibrium between pairs of loci were conducted using the index of association described by Brown et al. (1980) in the R package POPPR 1.1.4 (Kamvar et al. 2014). Linkage between loci within or among samples was used to assess independent assortment or if significant inbreeding occurred. Pairwise F_{ST} values across all loci were calculated in ARLEQUIN 3.3.2.2 (Excoffier and Lischer 2010). These pairwise F-statistics were used to determine within and among sample genetic variation to resolve potential sample relationships. Because there were a large number of individuals, loci, and tests performed, type I error using a standard p-value (alpha) would bias our interpretation of the results. To control the false discovery rate, an initial alpha of 0.05 was adjusted using the Benjamini-Yekutieli (2001) method (Online Resource 1).

Multivariate analyses were used to explore non-random patterns of genetic variability within and among samples in a semi-spatial context. The analysis was semi-spatial because no explicit spatial data were used; however, individuals were identified by the geographic group in which they were collected. Principal component analysis (PCA) was used to investigate covariance across individual oysters and between the individuals' allele calls using methods implemented by the R package ADEGENET 1.4.2 (Jombart 2008). For each column, the mean of the data was subtracted and divided by its standard deviation and missing allele calls were replaced with the column mean. There were 720 missing allele calls substituted out of 56,580 total allele calls due to failed fluorescence or ambiguous clustering (~1.3% missing data). The results of the PCA summarizes covariance between individual genotypic data and were used to examine differences on an individual, rather than on a population level. The advantage of PCA is that it does not make assumptions about the relationships within the data, such as HWE and linkage equilibrium, like the other population genetic analyses that were performed in this study.

Bayesian clustering methods were performed to determine the probable number of population clusters and to determine likely cluster membership for each oyster. Individual genotypes were clustered using STRUCTURE 2.3.4 (Pritchard et al. 2000), and were performed with correlated allele frequencies, admixture, and sampling location used as a prior. Simulations were run for 100,000 iterations with 50,000 initial steps discarded while k, number of putative populations, was set from one through ten at four iterations per k as recommended by the software developer. The most likely value of k was determined using the Δk method described by Evanno et al. (2005) and implemented in STRUCTURE HARVESTER (Earl and VonHoldt 2012). In addition, GENELAND 4.0.5 (Guillot et al.

2005) implemented in R was used to perform spatially-dependent clustering. Geographic coordinates were included as a prior distribution in the model and only samples that we were confident about their location were included in the spatial analyses. Four different subsets of the data were run for six iterations per set to account for possible cryptic regional population clusters and the highest mean log-likelihood of the data was used to determine the most likely run. GENELAND was run for 100,000 iterations with a thinning of 100, with k set from one to ten, and using the correlated allele frequencies model. The runs were post-processed by discarding the initial 200 iterations kept from the thinning. Both Structure and GENELAND use a similar Bayesian clustering algorithm that assumes putative populations conform to expected proportions of HWE and linkage equilibrium.

RESULTS

In total, 744 individuals were genotyped across 48 loci. Of these 48 loci, one was an analytical control and six were excluded due to poor fluorescence or ambiguous calls, leaving 41 markers for population genetic analysis (see Online Resource 1). Across all samples, expected heterozygosity ranged from 0.004 for locus prp-198 to 0.50 for locus cm4-346 (Table 1). Within samples, the NEH® sample had the lowest mean expected heterozygosity at 0.24, while the LR13 spat sample had the highest mean expected heterozygosity of 0.33. There were 14 loci that exhibited deviations from the expectations of Hardy-Weinberg Equilibrium (HWE) among the 41 loci (Table S1); however, no loci were out of HWE across all samples. Three loci, ba-83, nss1-228, and rpo-422, had significant deviations from HWE in seven, six, and five of the nine samples, respectively. Two loci, mych-289 and prp-198, were monomorphic in more than half of the samples (Table S1). Within samples, the number of loci out of HWE ranged from zero in LR to eight in the LR13 with a median of four loci out of HWE across all samples (Table S1). The linkage analysis indicated the reference samples LR, TI, and NEH® had significant global linkage ($p < 0.002$, $p < 0.001$, and $p < 0.001$, respectively, Table S2); however, no pairs of loci were significantly linked across all samples. The locus pairs nss-417 and hsp6-205, rpl9-451 and hsp6-205, and rpl9-451and nss-417 had large estimated linkage values indicating low probability of independent assortment in both the LR reference and LR13 spat samples; however, these pairs were not linked in any other samples. Additionally, the linked loci had significant heterozygote excesses in the LR13 sample ($p < 0.001$), but not the LR sample. A pairwise relatedness analysis demonstrated that LR13 had a higher than expected levels of relatedness compared to LR and LR14 (Figure S1).

Population pairwise F_{ST} values were summarized in Table 2. Adult oysters were sampled from multiple locations within the Lafayette, Elizabeth, and James Rivers and pairwise F_{ST} values were first calculated between

sampling locations within each river. We found that all within river comparisons were non-significant ($p > 0.01$), therefore within river adult samples were combined for all subsequent analyses. The F_{ST} values were significantly different between 26 out of 36 total pairwise comparisons ($p < 0.01$), and had a median value of 0.02 ranging from -0.04 ($p = 0.99$) between TI and RR and 0.14 ($p < 0.01$) between TI and NEH®. The F_{ST} values calculated between NEH® samples and all other samples were elevated relative to other pairwise comparisons, ranging from a minimum F_{ST} of 0.10 compared with LR14, ER, and JR to a maximum F_{ST} of 0.14 compared to both the RR and TI samples and all comparisons were significantly different ($p < 0.01$). The median F_{ST} for NEH® is 0.12 and the median of all other comparisons without NEH® is 0.01, an order of magnitude difference. The LR sample was significantly different from all other samples with median value of 0.01 with the exception of TI ($F_{ST} = -0.02$, $p = 1.00$). The spat samples LR13 and LR14 were significantly different from each other ($F_{ST} = 0.03$, $p < 0.01$). The LR13 spat were significantly different from the LR reference sample ($F_{ST} = 0.01$, $p = 0.01$) but not TI ($F_{ST} = -0.03$, $p = 1.00$). The LR14 spat were not significantly different from ER, JR, or TI ($F_{ST} \sim 0$, $p = 1.00$); however, they were significantly different from the LR reference sample ($F_{ST} = 0.01$, $p < 0.01$).

The PCA resolved several clusters of individuals. For reference group samples, the PCA indicated the presence of discrete clusters of individuals that were partially reflective of the geographic relationships of the samples in the Chesapeake Bay. The first two principal components retained from the PCA explained 6.5% and 4.8% of the variance in the individual genotypic data (Figure 2a). The NEH® individuals were separated from all other genotyped individuals with minimal overlap in component space. The individuals from ER, JR, and GR clustered together, while the majority of individuals from RR and TI clustered together. Individuals from LR had a wider distribution, situated in component space among several clusters including space occupied by RR and TI, and in component space occupied by ER, JR, and GR. The spat samples LR13 and LR14 were separated from each other with minimal overlap. The LR13 sample had a wider distribution occupying space primarily with LR and some with ER, JR, and GR. While LR14 had a narrower distribution, sharing component space primarily with ER, JR, and GR.

The results of the different STRUCTURE simulations had similar results and only the simulations using correlated allele frequencies and admixture are discussed. The results of the STRUCTURE simulations converged on three clusters (mean Ln likelihood = -25136) with individual oysters from RR and TI in cluster one; NEH® individuals exclusively in cluster two; while ER, JR, and GR individuals in cluster three (Figure 2b). The individual adults from LR were assigned to multiple clusters and showed evidence of admixture between clusters one and

three. The majority of LR individuals (60%, 32 of 53 oysters) were assigned to cluster three and the remainder were assigned to cluster one with the all RR and TI individuals. The majority of LR13 individuals (75%, 74 of 99 spat) were assigned to cluster one with RR and TI reference samples, while remaining LR13 spat were assigned to cluster one, which includes individuals sampled from the JR, ER, LR and GR. Unlike the LR13, which had individuals assigned to different clusters, the spat in the LR14 sample were exclusively assigned to cluster one. The STRUCTURE simulation results were qualitatively similar to the results from the PCA.

When spatial data was explicitly taken into account, simulations performed in GENELAND resolved four population clusters in the Chesapeake Bay when all individuals were included. Overall, these results are similar to the PCA and STRUCTURE results except that results from GENELAND put the RR and TI samples into separate clusters (Figure S2). The discrepancy between these results is likely due to the spatial data used as priors in the GENELAND model input. In a second analysis including only those individuals sampled within the Lafayette River (i.e., LR, LR13, and LR14), an upriver section, eastward away from the mouth of the Lafayette, contains a cluster with 100% of the individuals from the LR reference sample, 91% of the LR13, and 31% of the LR14 individual spat (Figure 3). A second cluster located downriver and including the mouth of the Lafayette, which is connected to the Elizabeth River, contains 69% of LR14 and 9% of LR13 individual spat.

Within the Lafayette River, the temporal and spatial patterns of post-larval oyster settlement based on the shell-string surveys contrasted with patterns of spat recruitment based on the spat-collectors. Within each year, the timing of settlement among shell-string sites was similar. Both the temporal and spatial patterns, however, varied between 2013 and 2014 (Figure S3). In the 2013 shell-string survey, post-larval oyster settlement was initially observed in mid-June and steadily increased with a peak in early August (Figure 4). By September of 2013, the supply of spat had decreased to levels that were observed in the beginning of the spawning in June. In 2014, the first post-larval oysters were not observed until mid-July, the peak of post-larval settlement occurred in early August, and a second peak was observed in early-September. The spatial pattern of settlement also differed between years (Table S3). In 2013 the majority of the post-larval oysters were recovered mid-river and upriver with few settling at the mouth, while in 2014 the majority of post-larval oysters were recovered near the mouth with some settlement upriver (Figure S3). The spatial pattern of recruitment was inferred based on the 2013 and 2014 spat-collector data. The total number of recruited spat in 2013 was 937 from 51 spat collectors (Figure 5a). The majority of the spat were collected near the mouth of the river (90%), very few were collected mid-river (6%) and up-river (4%). In

2014, the number of recruited spat was higher; 3132 spat were collected from 36 spat collectors (Figure 5b). Similar to 2013, the majority of recruited spat were from spat collectors returned from near the mouth of the river (94%), and few were collected from mid-river (4%) and up-river (2%).

DISCUSSION

We developed a SNP panel for use in a rapid, high-throughput platform that is easily scalable for genetic marker-based management studies of any species. Previous investigations of *C. virginica* to assess regional population connectivity predominantly relied on mtDNA or microsatellite markers (Milbury et al. 2004, Hare et al. 2006, Carlsson et al. 2008). Our use of SNPs overcame many of the limitations exhibited by other molecular markers, particularly those of microsatellites (Morin et al. 2004, Garvin et al. 2010). Oysters have high levels of intra-specific polymorphism, and as a result null alleles and homoplasy are pervasive problems when using microsatellites (Launey and Hedgecock 2001, McGoldrick et al. 2000, Reece et al. 2004). Furthermore, SNPs are easily transferable between laboratories because they are less susceptible to subjective interpretation of fluorescence peaks, which is a common problem with standard microsatellite techniques. The SNP panel developed for this study has utility in *C. virginica* aquaculture and fishery research context, including stock identification, monitoring of diversity, and inbreeding assessment.

Our results demonstrate that the SNP panel developed for this project can resolve genetic differences on small spatial scales, which is necessary for use in recruitment studies and restoration management applications. The SNP panel consisting of 41 loci resolved significant differences between adult oysters from the Lafayette, Elizabeth, and James Rivers. We expected *a priori* that there would be little difference between oysters from these sites because these rivers are hydrodynamic connected (Shen et al. 1999) and connectivity is within the time scale of oyster pelagic larval duration (Kennedy 1996). Additionally, our SNP panel resolved significant differences between oysters sampled from the lower Chesapeake Bay and those collected from the Rappahannock and Great Wicomico Rivers, which are mid-Bay and more than 70 km distant. Previous work using eight microsatellite markers resolved significant differences within the Chesapeake Bay, and demonstrated a subtle pattern of isolation by distance on spatial scales similar to the larger geographic scale encompassed by our study (Rose et al. 2006). The statistical power of the 41 SNPs used by this study is comparable to that of the eight microsatellites used by Rose et al. (2006) when trying to resolve moderate levels of population differentiation ($F_{ST} \sim 0.01$, Morin et al. 2009). Thus, our SNP panel was an adequate tool to evaluate annual oyster recruitment on small spatial scales.

The reproductive contribution of restoration oysters newly recruiting within the Lafayette River was assessed using the SNP panel developed for this study. Our panel was sufficient to resolve significant genetic differences between the hatchery-bred NEH® oysters and field-sampled oysters. However, neither NEH® offspring nor NEH®-hybrid spat were detected in the samples from the Lafayette in 2014 (Figure 2b). We do not interpret our results to suggest that the NEH® oysters introduced by CBF did not spawn nor contribute progeny during the time span of our study. Rather, it is likely NEH® oysters spawned, but our sampling did not capture NEH® derived spat. Approximately 114,000 NEH® oysters were planted in the Lafayette River in 2013 for this project, and the estimated census size is $3.4x10^9$ oysters in the James River, and $3.0x10^6$ oysters in the Elizabeth and Lafayette Rivers combined (Mann et al. 2015). The likelihood of detecting a genetic signal of NEH® progeny was low because the signal could be obscured by resident-oyster reproductive output. Wilbur et al. (2005) made a similar suggestion to explain an absence of a genetic signal from restoration scallops amidst signals from wild scallops in a Florida estuary. The Tangier Island oysters, the second restoration line used in this study, were not significantly different from the resident adult oysters sampled in the Lafayette, Elizabeth, or James Rivers. This lack of significant differentiation was not expected considering the geographic distance (greater than 100 km) between the lower Chesapeake Bay and Tangier Island; however, the history of restoration and oyster seed-stock movement throughout Chesapeake Bay provides a possible explanation. The CBF has been introducing oysters into the Lafayette and Elizabeth Rivers from a variety of sources around the Chesapeake Bay since 1999 (T. Leggett, personal communication). It is likely that significant genetic differences between oysters residing near Tangier Island and oysters in the lower Chesapeake Bay once existed due to isolation by distance (Rose et al. 2006) but may have been diluted out as a result of CBF restoration plantings.

Despite the paucity of data that the experimentally planted oysters contributed to the 2014 recruitment, our analyses reveal a potential influence of oysters genetically similar to the RR reference samples (Figure 2). According to records from the CBF, about 1.1 million Rappahannock oysters were introduced to the Tanner's Point and Larchmont reefs in the Lafayette River during the course of restoration activities in 2011 and 2012 (Table 3). Our analyses suggest that the Rappahannock oysters planted in the Lafayette reproductively contributed to the Lafayette genepool and influenced the genotypic signature for some of the 2013 spat in this study. However, variation in reproductive success can lead to an overestimation of the long-term contributions by restoration oysters (Hedgecock and Pudovkin 2011), and this may be the case with the Rappahannock oysters planted by the CBF.

Thus, while our results demonstrate a contribution of oysters that are genetically similar to RR, a sustained contribution of Rappahannock oysters cannot be inferred from this data. Therefore, a monitoring strategy to obtain cohorts from specific reproductive events over multiple years would be sufficient to parse interannual variation in larval demographics.

The results of our study suggest that there were variable sources for spat recruitment within the Lafayette River on interannual timescales. Spat collected in 2013 had genotypic signatures that were more closely related to reference oysters sampled from the Lafayette River, and the spat were significantly different from the reference samples collected in adjacent rivers. These results indicate that 2013 was a retentive year in which the prevailing reproductive contribution to recruitment was from within the Lafayette River and there was low dispersal of larvae produced in Elizabeth or James Rivers. In contrast, the spat collected in 2014 had a different genotypic signature similar to the reference oysters collected from the Elizabeth, and James Rivers. We interpret our results to suggest that the 2014 spat were a mixture of larvae spawned in the Lafayette and the adjacent rivers. The pattern of recruitment in which more spat are found near the mouth of the Lafayette was similar between years (Figure 5); however, the relative contribution of sources was different (Figure 3). Differences in the observed interannual source populations for the recruiting spat were likely a consequence of variance in reproductive success as well as variance in larval survival (Hedgecock 1982). For example, *C. virginica* are highly fecund, broadcast spawners whose pelagic larvae experience high mortality, and variations in these processes differentially impact annual reproductive success. As a result, variable contributions by different source populations to new recruits influences their resulting genotypic signatures (Hedgecock and Pudovkin 2011). The changes to reproductive success can be associated with intrinsic demographic variability such as fluctuations in fecundity, but other factors may also be important such as changes to the physical environment encountered during the spawning season.

To explore a potential physical mechanism for the observed variance in recruitment patterns and genotypic signatures between 2013 and 2014, wind data from Norfolk Naval Air Station, VA (Network ID: GHCND:USW00013750, obtained from NOAA National Climatic Data Center) were examined. Winds were considered because they are an important mechanism for physical mixing of relatively shallow water masses similar to the lower Chesapeake Bay. We found that wind patterns in the summer months before and during the peak post-larval settlement differed between 2013 and 2014 (Figure 4). In 2013, the prevailing winds blew from the south-southwest (Figures 4 and 5a), possibly forcing water out of the Elizabeth River into the James River near Newport

News, Virginia and creating a local sea-surface-height low in which water from the Lafayette River could move out in response to a horizontal pressure gradient. As surface waters in the Lafayette River moved out, oyster larvae would be dispersed, and larvae would possibly be restricted from moving into the Lafayette except in deeper tidal flow. However, bivalve larvae in laboratory settings demonstrate vertical movement in response to steep salinity gradients, restricting larval distribution below the halocline and limiting deeper, tidal dispersal (Mann et al. 1991). Alternatively, winds out of the southwest could have forced surface waters toward the Lafayette River creating a local sea-surface-height high, restricting larval dispersal due to reduced surface advection against a horizontal pressure gradient, and forcing locally produced larvae to settle within the river.

In contrast, 2014 exhibited different wind patterns the weeks before and during peak post-larval settlement (Figures 4 and 5b). The winds were weaker on average and coming from both the south-southwest and east-northeast in July and August, respectively. Furthermore, the weekly zonal winds (u, Figure 4) in July and August 2014 demonstrated an alternating east-to-west pattern that was distinct from the autocorrelated pattern in 2013 for the same months (Figure 4). The shifting winds in 2014 likely facilitated mixing of local water masses and concurrent planktonic larvae contributing to the genotypic signal observed for the 2014 spat. The mesohaline James River tidal front weakens during ebb tide and mixes with waters from the polyhaline Elizabeth and Lafayette Rivers (Shen et al. 1999). The mixing facilitates expansion and dispersal of phytoplankton blooms initiated in either the Lafayette, Elizabeth, or James Rivers across this region in about 20 days (Mulholland et al. 2009, Morse et al. 2011). The temporal scale of the circulation patterns within the lower Chesapeake Bay is well within the pelagic larval duration of oysters (Kennedy 1996) and oyster reef connectivity between these rivers is a reasonable hypothesis. We suggest that the contribution of oysters spawned in disparate regions of the lower Chesapeake Bay to annual oyster recruitment in the Lafayette River varies on interannual time scales and is partially dependent upon the winds during the summer spawning season. Future work should investigate the interaction of wind forcing, hydrodynamics, and larval dispersal and settlement in this region.

During peak oyster spawning months in the summer, the Chesapeake Bay can be host to several potentially harmful algal species. The harmful algal bloom (HAB) events in the lower Chesapeake Bay during 2013 and 2014 were different. Specifically, the duration and geographic extent of the annual bloom of *Margalefidinium* (previously known as *Cochlodinium) polykrikoides* varied between these two years. In 2013, the *M. polykrikoides* bloom was first observed in the Lafayette on August 9th and lasted in the James, Elizabeth and Lafayette Rivers region through

September 6th. The effects of *M. polykrikoides* on adult and larval oysters in the field are not well understood. Laboratory studies, however, have demonstrated that adult oysters will close their shells to possibly reduce toxin exposure (Hégaret et al. 2007). Larval oysters exposed to *M. polykrikoides* in laboratory studies have demonstrated increased mortality possibly due to factors including toxicity to HAB by-products (Tang and Gobler 2009, Mulholland et al. 2009, Reece et al. 2012). It is possible that the 2013 bloom either restricted further spawning as adults limited exposure by closing their shells or that larvae in the plankton suffered high mortalities reducing the supply of oyster larvae into the Lafayette mid-August through early September. In contrast during 2014, the *M. polykrikoides* bloom in this region was limited both the spatial and temporal extent (Reece 2014). Few HAB samples were collected in the region from small bloom patches in mid-June through early July (Reece 2013, 2014, Marshall and Egerton 2013, 2014), before the peak timing of post-larval settlement within the river (Figure 4). The reduced HAB activity might have been partially contributed for the overall increased spat recruitment in 2014 as quantified by the spat collectors (Figure 5b). Unfortunately, our data cannot resolve the dynamics between timing of larval settlement, spat source, and HAB activity in the region; however, our results provide a tantalizing suggestion for recruitment control by HABs in the lower Chesapeake Bay that deserves further study.

Our results offer support for larval settlement projections produced by the Sisson and Shen (2012) connectivity model. Overall, the model output demonstrates that oyster larvae spawned in the Lafayette River will settle out in the upper parts of the river away from the mouth leading to the rest of the lower Chesapeake Bay (Figure S4). The results from 2013 exhibit a similar retentive capacity of the Lafayette such that larvae produced in the river can settle within the river (Figure 3). A limitation of our study was that no spat collectors were placed outside the Lafayette to identify dispersal out of the river; however, several spat collectors in both 2013 and 2014 were sampled at the mouth of the Lafayette. The results of the collectors at the mouth demonstrate genotypic signatures that were both similar to the Lafayette reference sample in 2013 and the adjacent rivers, while the 2014 spat collector results at the mouth had a more cosmopolitan genotypic signature. The explanation for the discrepancy between the model projections and the field results were likely due to several factors. For example, the model was initialized with winds from 2008, a year with wind patterns more similar to those observed in 2013. The model cannot represent the wind variability present both in 2013 and 2014. Because the winds in 2013 were more similar to 2008 used to initialize the model, the observed retentive capacity of the Lafayette during 2013 compares well with the expected settlement projections produced by the model. In addition, the model did not simulate the settlement of

larvae produced outside the Lafayette River, and as a result the relative contribution by different rivers in the Lower Chesapeake Bay to the Lafayette cannot be assessed from the model output. Lastly, the connectivity model does not explicitly represent conditions within the estuary important for larval survival, settlement, and spat recruitment thus limiting the comparisons between observations and model output. In reality, the regions of highest recruitment projected by the model (Figure S4), upriver away from the mouth, are also the areas that have the lowest recovery per spat-collector (Figure 5). The upriver regions are not suitable for spat survival because these areas have little hard substrate for attachment, suspended sediments from runoff smother spat, and high heat during low tides in the summer spawning season easily desiccates spat.

CONCLUSIONS

Restoration projects, which introduce live organisms, make the assumption that planted individuals will add to the spawning potential of local populations and contribute to subsequent recruitment. The overall aim of this study was to test whether restoration efforts impacted the annual oyster recruitment such that genetic contributions could be detected within the Lafayette River, VA. Our study failed to detect any contribution in newly recruiting oysters from the two experimentally planted oyster strains chosen to be easily identified using molecular genetic methods. We did find evidence to suggest that oysters from the Rappahannock River previously planted for restoration had contributed to recruitment within the Lafayette River. The geographic distance between these rivers precludes direct dispersal, and as a result we can conclude that the observed spat genotypes are due to restoration activities. Furthermore, our research demonstrated that the source populations for newly recruiting oyster spat varies on interannual timescales within the Lafayette River. Thus, we suggest that ecological investigations for management objectives into sessile, broadcast-spawning estuarine species should consider the interannual differences in larval dispersal and recruitment.

We identified several potential factors influencing the source and magnitude of oyster settlement and recruitment within the Lafayette River. Due to the timing of the blooms, HABs in 2013 could not influence the post-larval oysters settling in the Lafayette until the second week of August. It is more likely that the winds influenced the hydrodynamics, precluding significant contributions of larvae spawned outside the Lafayette; however, some 2013 spat were similar to oysters from outside the Lafayette. It is possible that the hydrodynamics in 2013 were sufficient for oysters spawned outside the Lafayette to settle in the Lafayette, but HABs may have exerted a greater control on post-larval survival in the late summer. In comparison, HABs in 2014 were early and limited in extent,

464 while shifting zonal winds provided ample energy to physically mix the water masses and the resident plankton in
465 the lower Chesapeake Bay. Observations of the timing of spawning in the lower Chesapeake Bay, identification of
466 specific cohort settlement timing, and plankton community composition would help to resolve the dynamics
467 between oyster larval survival and HABs. We do not to intimate that the factors mentioned are the only influences,
468 nor the most influential for settlement and recruitment; however, the preponderance of data lead us to suggest that
469 the potential linkages mentioned are relevant on interannual timescales.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Data availability: The datasets generated by this study are available from corresponding author upon request.

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FIGURES

Fig. 1 (a) Locations within the Chesapeake Bay where oyster samples were collected. G – Great Wicomico River; T – Tangier Island; D – Deepwater Shoal, James River; W – Wreck Shoals, James River; C – Cruisers Rock, James River; B – Eastern Branch, Elizabeth River; H – Hospital Point, Elizabeth River; L – Lafayette River. (b) Lafayette River inset with shell-string locations labeled with S and river regions labeled

TITLE: Multiple drivers of interannual oyster settlement and recruitment in the lower Chesapeake Bay
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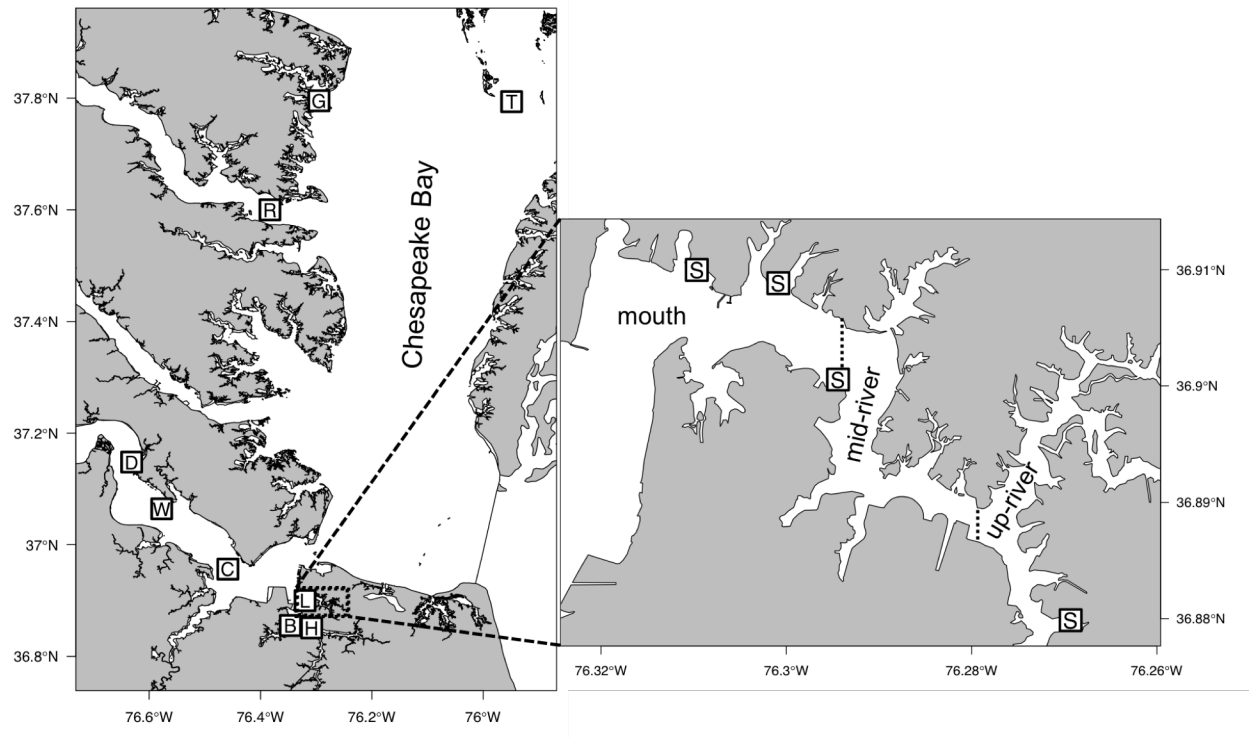
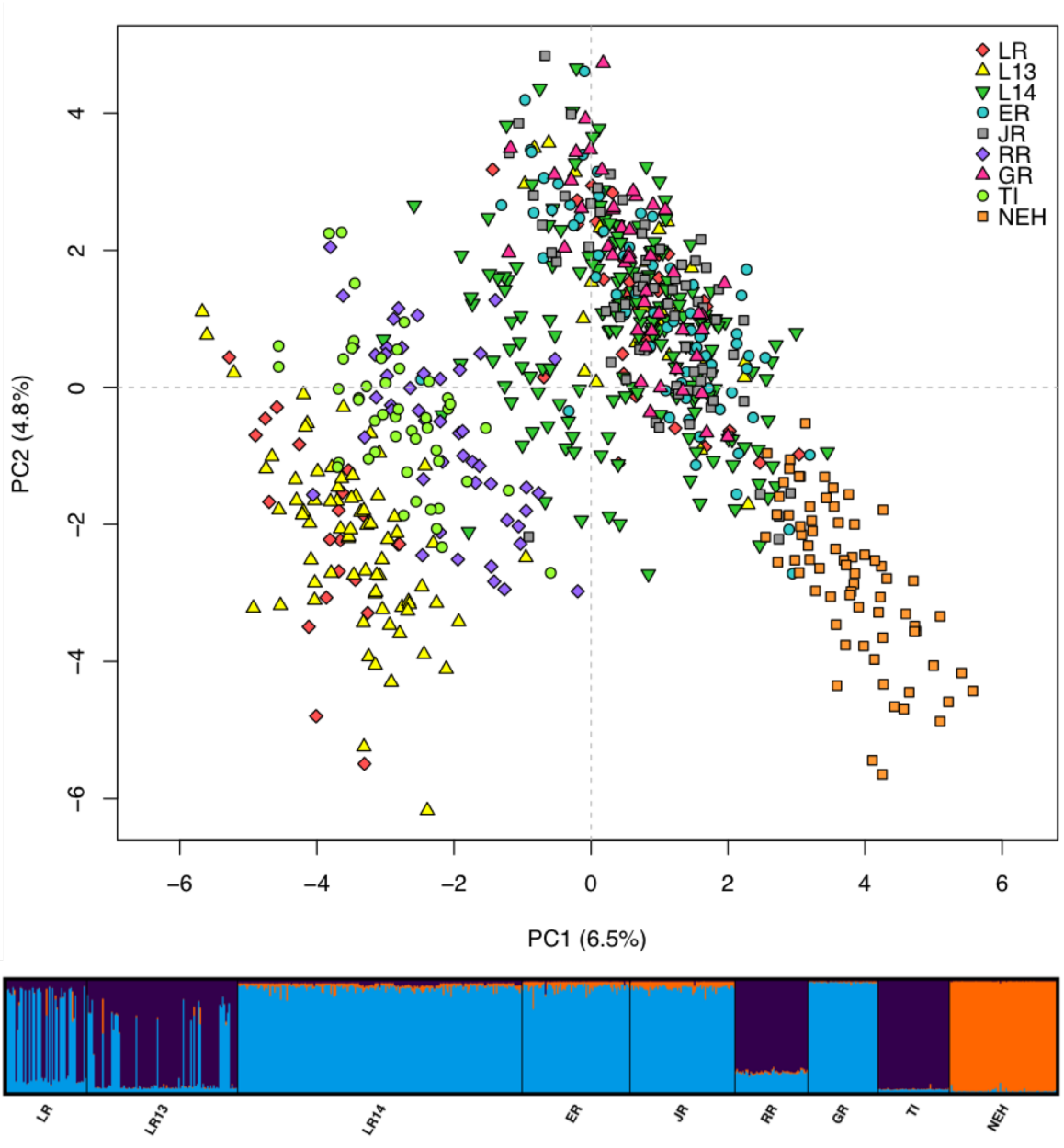
Figure 1



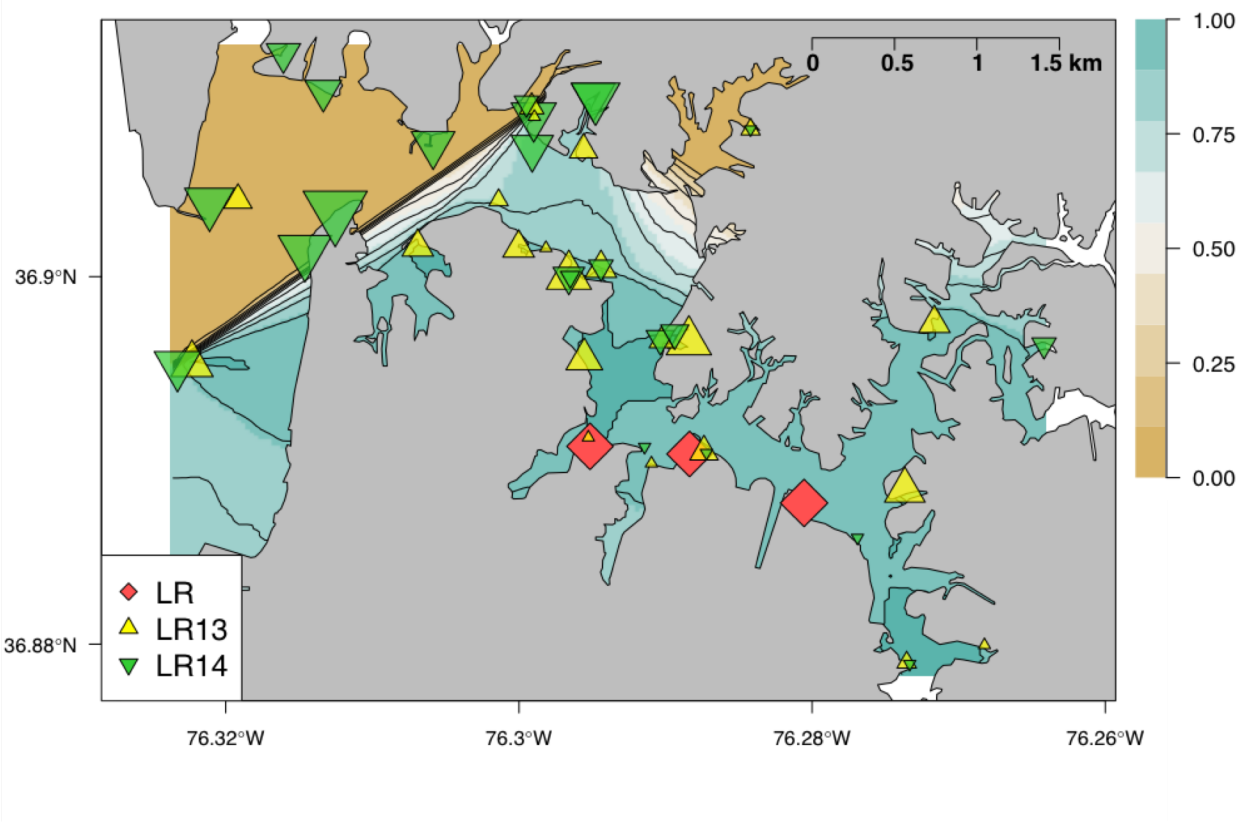
599 **Fig. 2** (a) Principal Component Analysis results for samples grouped by sampling group. Each point represents one
600 individual oyster genotype. Variance explained for principal component one and two are in parenthesis on x and y
601 axes, respectively. (b) Structure analysis results where three clusters (k = 3) is the most likely result; vertical bars
602 indicate posterior probabilities of cluster membership for individual oyster genotypes. Cluster designation starts at
603 the top and moves down; cluster one is indicated in purple; cluster two is indicated in orange; and cluster three is
604 indicated in blue. For these results, admixture was allowed, allele frequencies were correlated, and sampling
605 locations were used as a prior. Figure produced using Clumpak (Kopelman et al. 2015)

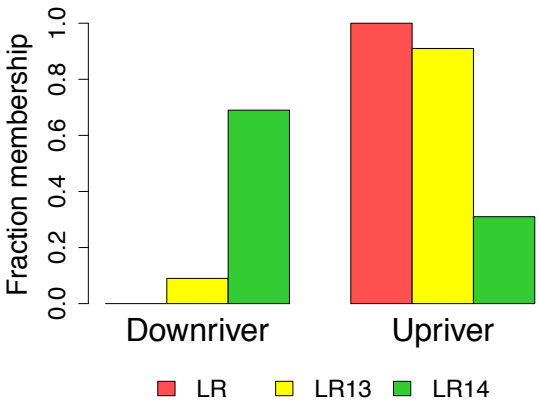
Figure 2


LR
L13
L14
ER
JR
RR
GR
TI
NEH
PC2 (4.8%)
PC1 (6.5%)
−6
−4
−2
0
2
4
6
LR
LR13
LR14
ER
JR
RR
GR
TI
NEH

606 **Fig. 3** (a) Results from GENELAND analysis for *Crassostrea virginica* sampled from the Lafayette River, VA.
607 Colored contours represent probability of belonging to downriver cluster (west toward the mouth) or to the upriver
608 cluster (east toward the head). Adult samples are represented by red diamonds, spat from 2013 are represented by
609 yellow upward pointing triangles, and spat from 2014 are represented by green downward pointing triangles. The
610 size of the symbol for LR13 and LR14 are proportional to the number of samples genotyped from each location. The
611 colorbar on the right is the posterior probability of individuals being assigned to the first cluster. (b) GENELAND
612 results displaying proportion of membership for Lafayette adults, 2013 spat, and 2014 spat to the downriver and
613 upriver clusters

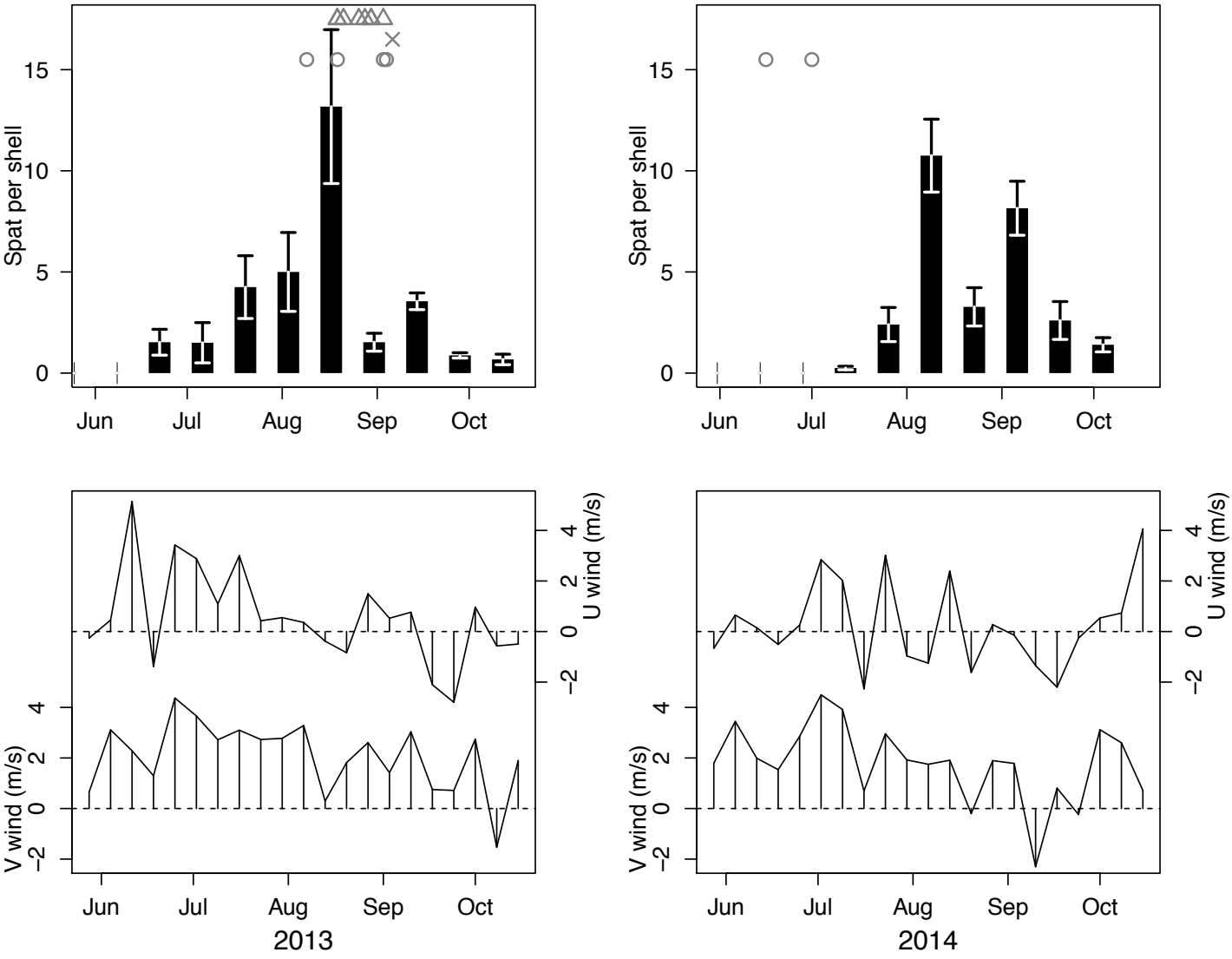
Figure 3


0
0.5
1
1.5 km
1.00
0.75
0.50
0.25
0.00
36.9°N
36.88°N
LR
LR13
LR14
76.32°W
76.3°W
76.28°W
76.26°W


Fraction membership
0.0
0.2
0.4
0.6
0.8
1.0
Downriver
Upriver
LR
LR13
LR14

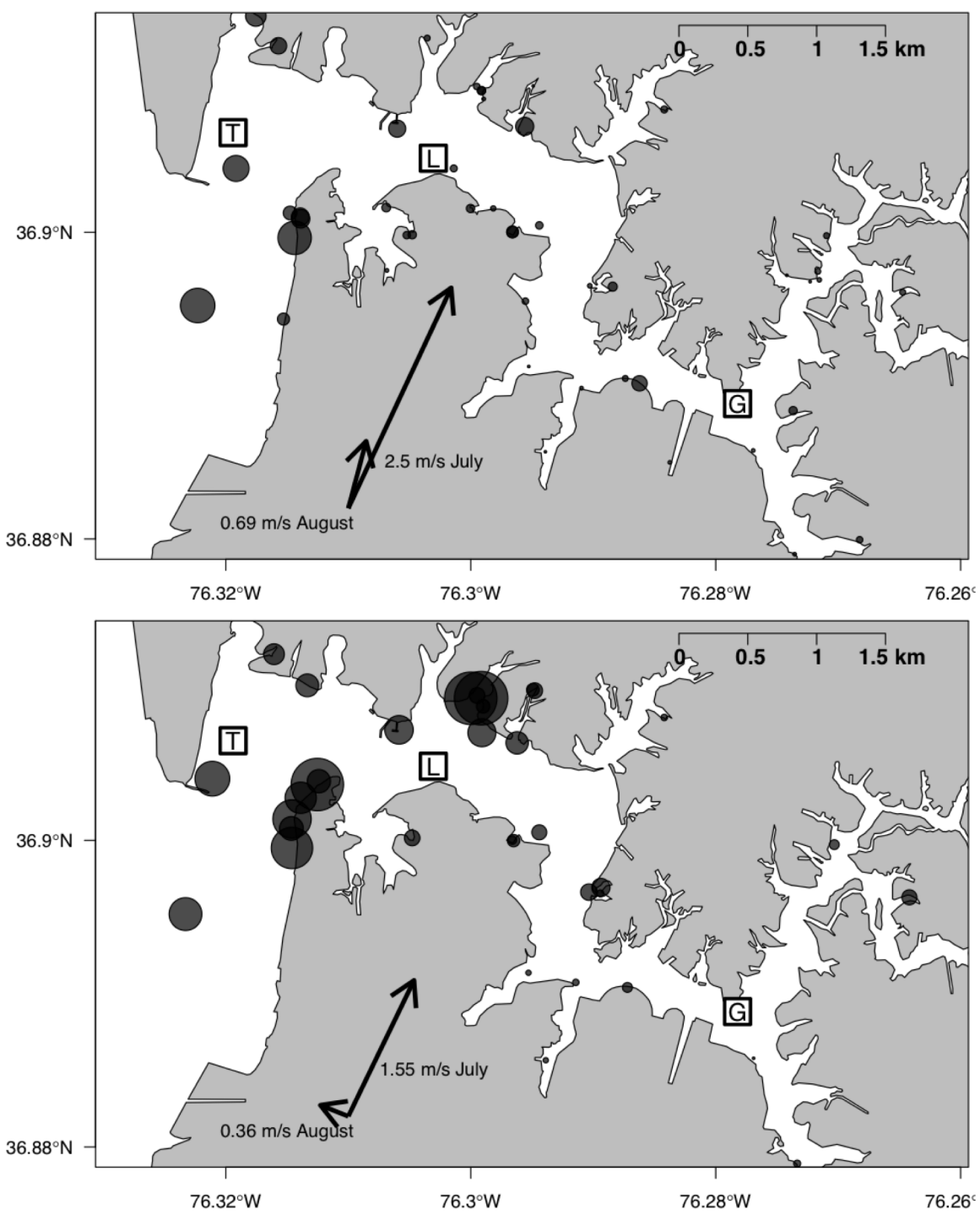
614 **Fig. 4** Top row plots display the number of spat per shell from shell-string surveys in 2013 (left column) and 2014
615 (right column). The error bars indicate standard error. Gray symbols near the top of the plots indicate the timing of
616 harmful algal bloom events where circles were events reported in the Lafayette River, the X was an event reported
617 in the Elizabeth River, and triangles were events reported in the James River. The bottom row plots show U winds,
618 which were weekly averaged zonal winds where positive were winds blowing to the east and negative were to the
619 west, and V winds, which were weekly averaged meridional winds where positive were winds blowing to the north
620 and negative were to the south. Data were observed winds at Norfolk Naval Air Station, VA (Network ID:
621 GHCND:USW00013750, obtained from NOAA National Climatic Data Center)

Figure 4


Spat per shell
0
5
10
15
Jun
Jul
Aug
Sep
Oct
Spat per shell
0
5
10
15
Jun
Jul
Aug
Sep
Oct
V wind (m/s)
−2
0
2
4
U wind (m/s)
−2
0
2
4
Jun
Jul
Aug
Sep
Oct
2013
V wind (m/s)
−2
0
2
4
U wind (m/s)
−2
0
2
4
Jun
Jul
Aug
Sep
Oct
2014

622 **Fig. 5** (a) Total spat in 2013 from spat collectors deployed in the Lafayette River by citizen scientists. Monthly
623 averaged wind vectors for July and August are from Norfolk Naval Airbase. Size of symbol is proportional to
624 number of spat recovered at each station. Restoration Reef locations for deployment of experimental oysters are
625 denoted L for Larchmont (NEH® plantings) and G for Granby (Tangier plantings). In addition, the location of
626 Tanner's Reef is labeled with a T. (b) Total spat in 2013 from spat collectors deployed in the Lafayette River by
627 citizen scientists. Observed monthly averaged wind vectors for July and August are from Norfolk Naval Air Station,
628 VA (Network ID: GHCND:USW00013750, obtained from NOAA National Climatic Data Center)

Figure 5


0
0.5
1
1.5 km
T
L
G
36.9°N
36.88°N
2.5 m/s July
0.69 m/s August
76.32°W
76.3°W
76.28°W
1.55 m/s July
0.36 m/s August

TABLES

Table 1 HWE summary statistics for all samples. Statistics for observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (F_{IS}), and minor allele frequencies (MAF) were calculated over all loci per sample. Median, maximum, minimum and standard deviation (sd) across all loci per sample are displayed. The number of individuals (n) per sample are included

Sample		Ho	He	F_{IS}	MAF
LR	median	0.32	0.32	0.02	0.20
	max	0.60	0.51	1.00	0.50
	min	0.29	0.31	0.07	0.22
	sd	0.17	0.17	0.24	0.15
	n = 53				
LR13	median	0.28	0.34	0.00	0.22
	max	0.74	0.50	1.00	0.49
	min	0.00	0.01	-0.58	0.01
	sd	0.20	0.16	0.33	0.15
	n = 99				
LR14	median	0.29	0.32	0.00	0.20
	max	0.52	0.50	1.00	0.50
	min	0.00	0.00	-0.14	0.00
	sd	0.17	0.18	0.26	0.16
	n = 187				
ER	median	0.27	0.35	0.10	0.23
	max	0.52	0.50	0.80	0.49
	min	0.00	0.00	-0.15	0.00
	sd	0.17	0.18	0.24	0.17
	n = 71				
JR	median	0.30	0.38	0.00	0.25
	max	0.62	0.50	0.67	0.49
	min	0.00	0.00	-0.23	0.00
	sd	0.19	0.19	0.23	0.17
	n = 69				
RR	median	0.32	0.37	-0.04	0.24
	max	1.00	0.51	0.54	0.50
	min	0.00	0.00	-1.00	0.00
	sd	0.25	0.17	0.32	0.17
	n = 48				
GR	median	0.28	0.33	0.02	0.21
	max	0.57	0.50	0.56	0.50
	min	0.00	0.00	-0.15	0.00
	sd	0.17	0.18	0.19	0.15
	n = 46				
TI	median	0.33	0.34	-0.02	0.22
	max	1.00	0.51	0.66	0.50
	min	0.00	0.00	-1.00	0.00
	sd	0.25	0.18	0.34	0.16
	n = 47				
NEH®	median	0.13	0.18	-0.02	0.10
	max	0.64	0.50	0.84	0.49
	min	0.00	0.00	-0.30	0.00
	sd	0.22	0.21	0.28	0.18

	n = 70				

634

635 **Table 2** Pairwise F_{ST} values for oyster samples. The upper half of the matrix are p-values where bold underlined
636 values are significant ($p < 0.01$, Benjamini-Yekutieli corrected p-value). The lower half of the matrix are the
637 pairwise F_{ST} values with significant values bolded and underlined

	LR	LR13	LR14	ER	JR	RR	GR	TI	NEH®
LR		**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	**0.00**
LR13	**0.01**		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	**0.00**
LR14	**0.01**	**0.03**		0.40	0.55	**0.00**	**0.00**	1.00	**0.00**
ER	**0.01**	**0.03**	0.00		0.96	**0.00**	**0.01**	0.34	**0.00**
JR	**0.01**	**0.03**	0.00	0.00		**0.00**	**0.00**	0.32	**0.00**
RR	**0.02**	**0.01**	**0.03**	**0.04**	**0.04**		**0.00**	1.00	**0.00**
GR	**0.02**	**0.04**	**0.01**	**0.01**	**0.01**	**0.05**		0.71	**0.00**
TI	-0.02	-0.03	-0.01	0.00	0.00	-0.04	0.00		**0.00**
NEH®	**0.12**	**0.12**	**0.10**	**0.10**	**0.10**	**0.14**	**0.13**	**0.14**	

638

639 **Table 3** Oysters planted by the Chesapeake Bay Foundation into the Lafayette River

Year	Location, Lafayette River	Origin	Number	Method
2011	Larchmont Reef	Rappahannock	397,012	spat on shell
2012	Tanner's Point	Rappahannock	727,145	spat on shell
2013	Granby Reef	Tangier Island	1,800,000	spat on shell
2013	Larchmont Reef	NEH®	114,000	cultchless

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Electronic Supplementary Material 1: Details concerning the SNP panel development, significance testing correction, and connectivity model. In addition, supplementary figures are included. Figure S1 are results from relatedness analysis. Figure S2 are results from shell-string survey. Figure S3 are depictions of the connectivity model output. Supplementary table 1 displays across sample Hardy-Weinberg statistics results, table S2 are linkage analysis results, and table S3 are ANOVA results of shell-string data.

Electronic Supplementary Material 2: Table listing the single nucleotide polymorphism markers used for this study including marker names, putative gene, base pair alternates, literature reference, Genebank accession number, and primer sequences used for methods.