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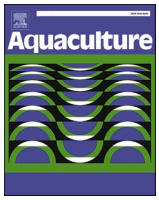

Relative importance of phenotypic plasticity and carryover effects in response to small salinity shifts during oyster aquaculture production

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ABSTRACT

Variability of water conditions in coastal environments could affect oyster aquaculture production through three main environmental-tolerance mechanisms: phenotypic plasticity, within-generation carryover effects, and selective mortality. Aquaculture production of eastern oyster, *Crassostrea virginica*, larvae occurs on weekly timescales in Virginia, with variations in salinity experienced by subsequent larval cohorts. The present study examined the relative importance of within-generation carryover effects – phenotypic changes during a previous life stage that impact a later stage – and phenotypic plasticity in shaping the performance of juvenile oysters after an experience of small differences in salinity (< 2 salinity units) during the larval stage. Genetic diversity was also assessed to rule out large shifts in allele frequencies, or loss of diversity, that would suggest observed effects were the result of selective mortality rather than carryover effects or phenotypic plasticity. Larval oysters were reared through settlement and metamorphosis under two salinities (thirteen and fifteen) that represent small differences between consecutive spawns in a hatchery. Juveniles were then raised in situ in two Virginia tributaries of the lower Chesapeake Bay, the York and Rappahannock rivers. Oyster production occurs within these two tributaries under distinct salinity conditions, with the Rappahannock tending to be of lower salinity. Metrics of survival, growth, oxidative stress, and condition index were compared to assess phenotypic plasticity and within-generation carryover effects. Juvenile oyster survival and physiology correlated with in situ environmental conditions rather than previous larval salinity experience. Specifically, juvenile oysters raised in the Rappahannock River had greater survival (13%), shell length (14%), condition index (38%), and dry tissue weight (78%) than those raised in the York River, regardless of larval salinity. Rappahannock River oysters also had 20% lower total antioxidant capacity than York River oysters. Genetic diversity remained high with no large shifts in allelic frequencies that would suggest non-random loss of alleles attributable to selection. Our results suggest that small salinity differences experienced in shellfish hatcheries 48 h after fertilization likely do not impact juvenile oyster performance during grow-out; rather, phenotypic plasticity likely underpins juvenile oyster performance during the transition from hatchery to farms. The importance of phenotypic plasticity presents another reason why farm site selection is critical to the performance and success of aquaculture product. Future studies are needed to further identify whether larval responses to salinity conditions are dependent on additional environmentally relevant conditions like temperature or the timing of exposure post-fertilization to better understand the relative importance of phenotypic plasticity, within-generation carryover effects, and selective mortality within oyster aquaculture.

1. Introduction

Aquaculture of the eastern oyster, *Crassostrea virginica* (Gmelin, 1791), occurs in highly variable coastal environments along the East and Gulf coasts of the United States where oysters routinely encounter challenging environmental conditions (Proestou et al., 2016; Shumway, 1996). During hatchery production where spawning occurs weekly, temporal changes in water conditions may produce physiological and genetic differences between cohorts of larvae that could impact production on farms. Depending on magnitude and duration, changes in environmental conditions can have cascading impacts from the molecular to whole-organism levels that can last beyond a single life stage due

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to within-generation carryover effects – phenotypic changes from a previous life stage experience that impact a later stage – or selective mortality (Petitjean et al., 2019; Sokolova et al., 2012). Alternatively, phenotypic plasticity may enable larvae to maintain performance under a range of environmental conditions due to the production of multiple phenotypes from one genotype (Pigliucci, 2001). As mechanisms of environmental tolerance, within-generation carryover effects and selective mortality could negatively impact aquaculture production of the eastern oyster through declines in organismal performance (e.g., slower growth rates and therefore time to market) and genetic diversity, respectively. Alternatively, phenotypic plasticity may help sustain production through continued performance of oysters regardless of environmental conditions. While other environmental-tolerance mechanisms could impact oyster performance, the present study focuses on the three mechanisms previously mentioned. The relative influence of phenotypic plasticity, carryover effects, and selective mortality within the context of shellfish aquaculture and present-day variations in environmental conditions is currently unknown.

Throughout the hatchery process and the transition of oysters from hatchery to farm, there are multiple points when oysters may experience stress due to a rapid change in their environment. Salinity varies spatially and temporally in coastal environments where oyster aquaculture takes place (Muhling et al., 2018), often differing weekly between successive larval cohorts throughout a production season (Fig. 1), as well as between larval rearing conditions and farm environments. Hatcheries are not able to efficiently manipulate or control for salinity in production facilities, making fluctuations in salinity challenging for hatcheries to manage. Salinity is also a well-known environmental stressor to oysters. Changes in salinity can affect oyster development, physiology, and genetics by interrupting cell signaling and cellular processes due to changes in cell volume (Casas et al., 2018a; Davis, 1958; Hand and Stickle, 1977; Méthé et al., 2015a; Shumway, 1996). The lack of sophisticated osmoregulation in oysters makes salinity a suitable environmental factor for examining the relative influence of phenotypic plasticity, carryover effects, and selective mortality on oyster performance.

Of the three environmental-tolerance mechanisms of focus, phenotypic plasticity is likely more beneficial to oyster aquaculture production as a mechanism of resilience to present-day environmental variability and future climate change via rapid acclimatization (Hofmann and Todgham, 2010; Putnam et al., 2016; Seebacher et al., 2015). As a means of interacting with and responding to surrounding environments, phenotypic plasticity is ubiquitous among taxa and is expressed at almost all biological scales (Pigliucci, 2001). Plasticity can be categorized as developmental or labile (Eierman and Hare, 2016), with labile being the focus of this study because developmental plasticity overlaps with carryover effects in the present context. Labile plasticity, henceforth referred to as phenotypic plasticity, allows organisms to maintain homeostasis under stress throughout their lifespan and therefore resist loss of basic function during rapid changes in their environment (Lande, 2014; Putnam et al., 2016; Smith et al., 2013). One way to observe the occurrence of phenotypic plasticity is to monitor the performance of organisms before and after a transition to another environment, assessing various physiological metrics like growth and stress response molecules (Méthé et al., 2015a) to see if physiological performance is able to recover after the transplant (Barbosa et al., 2022). Oysters have a high degree of phenotypic plasticity, likely evolved from living in highly variable environments as sessile organisms (Robinson et al., 2014; Shumway, 1996). For example, oysters collected from different environments (i.e., reef locations) maintained osmotic performance when exposed to various salinity conditions, and while all oysters expressed some level of plasticity, the magnitude was dependent on the previous-environment to current-environment combinations (Eierman and Hare, 2016). The ability for oysters to effectively respond to an array of environmental conditions via phenotypic plasticity lies within their abundant stress-response genes that have overlapping functions and genetic duplications (Guo et al., 2015; Modak et al., 2021; Wang et al., 2012), which is likely beneficial to aquaculture production.

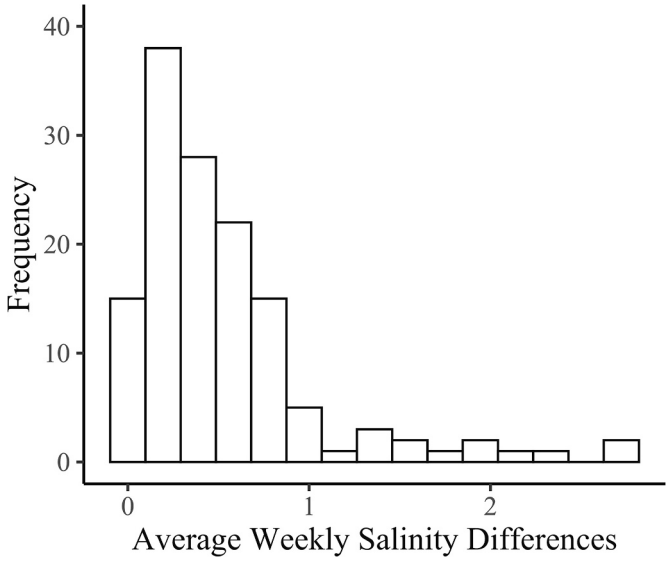

Fig. 1. Frequency of differences in average salinity (7 days) between consecutive weeks during the hatchery season (March–September) from 2015 to 2018. Salinity data are from National Oceanic and Atmospheric Administration (NOAA)'s Chesapeake Bay Interpretive Buoy System (CBIB)'s Stingray Point buoy at the mouth of the Rappahannock River, Virginia because of its proximity to our commercial hatchery partner (https://buoybay.noaa.gov/locations/stingray-point).

Experience of varied environmental conditions during an oyster's early life history can have latent phenotypic consequences impacting performance, referred to as within-generation carryover effects (henceforth carryover effects; O'connor et al., 2014). Carryover effects have been identified in mammals (Skibiel et al., 2022), birds (Steenweg et al., 2022), amphibians (Le Sage et al., 2022), fish (Jonsson and Jonsson, 2014), insects (Ezeakacha and Yee, 2019), and marine invertebrates (Hettinger et al., 2012; Richardson and Allen, 2023; Shen et al., 2022). In marine bivalves specifically, larval experience of ocean acidification conditions carried over and negatively affected shell and tissue growth (Hettinger et al., 2013, 2012) and metabolism (Zhao et al., 2019) of juveniles, while early juvenile exposure to ocean warming and hypoxia carried over to negatively impact early tissue and shell growth of adults (Donelan et al., 2021). At present, carryover effect studies on bivalves have mainly focused on global climate change stressors, like ocean acidification and temperature (Goncalves et al., 2016; Parker et al., 2015; Ross et al., 2016; Spencer et al., 2020). A few have focused on local stressors, like salinity, where beneficial carryover effects were identified with individuals previously in low-salinity conditions having the fastest growth rates once in juvenile environments (Manuel et al., 2023).

The underlying mechanisms responsible for carryover effects, while not well understood, may be linked to shifts in energy allocation (Hettinger et al., 2013, 2012; Pechenik et al., 1998). When organisms experience moderate environmental stress, they begin to allocate more energy towards cellular processes necessary for survival, negatively impacting other aspects of their biology, like growth and reproduction (Petitjean et al., 2019; Sokolova et al., 2012). Carryover effects likely stem from these costly metabolic shifts, especially when they occur prior to an energetically intensive life-stage transition, like metamorphosis, resulting in the next life stage beginning with an energy deficit (Pechenik et al., 1998). An alternative hypothesis for mechanisms of carryover effects that are not directly tied to energy deficits is epigenetic modification. While there is limited evidence with respect to marine organisms, epigenetic changes due to environmental experience could

underpin within-generation carryover effects (Eirin-Lopez and Putnam, 2019).

Environmental stress can lead to selective mortality, or the non-random mortality of individuals with phenotypes and underlying genotypes that are disadvantageous under the stressful conditions. Selective mortality is generally beneficial for the persistence of wild populations (Darwin, 2017; Gardner and Skibinski, 1991). Oysters are r-selected species, organisms that have high fecundity and little investment per offspring to ensure the continuance of the population regardless of high mortality, which could occur because of unpredictable environmental conditions. While survival in hatcheries is likely higher than in the wild, genetic diversity is already lower in selectively bred broodstock populations compared to wild populations, increasing the vulnerability of aquaculture production to further decreases in genetic diversity from selective mortality (Araki and Schmid, 2010; Lind et al., 2008; Varney and Wilbur, 2020; Zhang et al., 2017). Lower genetic diversity and smaller effective population sizes in selectively bred populations can also lead to higher levels of lethal mutations, or inbreeding depressions, further decreasing genetic diversity and effective population size (Gamfeldt and Källström, 2007; Plough, 2018; Plough et al., 2016). Although oysters produced for aquaculture are often selectively bred to thrive in certain environmental conditions, inbreeding depression may become exacerbated in the presence of additional environmental stressors, leading to a lower likelihood of survival in later life stages (Gamfeldt and Källström, 2007; Plough, 2012). Within oyster aquaculture, higher mortality could leave hatcheries and farmers with not only decreased production, but also the possibility of complete production loss from unexpected environmental stress.

The aim of this study was to examine the relative importance of environmental-tolerance mechanisms in shaping the performance of eastern oysters, *C. virginica*, through the hatchery-to-farm transition in oyster aquaculture. To study the role of these environmental-tolerance mechanisms on oyster aquaculture production, we utilized small differences in salinity conditions ($\leq$ 2 salinity units) at the hatchery stage to make sure results are relevant for standard hatchery operation. In the mesohaline region of the Chesapeake Bay where oyster aquaculture and hatchery production are common (see Fig. 3 in Beckensteiner et al., 2020), salinity frequently differs by less than one unit per week on average (Fig. 1). Though eastern oysters reside over a wide range of salinities due to their vast biogeographic range (5–40; [Shumway, 1996]), individual populations have more limited tolerances that affect their ability to withstand shifts in salinity (Eierman and Hare, 2013; Scharping et al., 2019; Swam et al., 2022). Larval oysters are more detrimentally sensitive to small changes in salinity (~2) at the low end of their tolerance range, while more capable of withstanding larger differences within their optimal salinity range (Scharping et al., 2019). Since salinity varies on a fine scale between weekly larval cohort production in hatcheries within mesohaline regions (Fig. 1), selective mortality in response to these small differences is unlikely if average salinity conditions are within the optimal range of 10–17 (McFarland et al., 2022; Scharping et al., 2019). Consequently, it was hypothesized that there would be no selective mortality associated with the small differences in salinity used in the experimental design.

Specifically, the relative importance of carryover effects and phenotypic plasticity in shaping the performance of juvenile eastern oysters, *C. virginica*, after experiencing different environmental conditions as larvae was examined. To assess the relative influence of these environmental-tolerance mechanisms on oyster aquaculture production, metrics of survival, growth, oxidative stress, and condition index were measured. Genetic diversity was coarsely monitored for the occurrence of large shifts in allele frequencies to confirm that selective mortality associated with the experimental design did not occur. In the absence of selective mortality, carryover effects were hypothesized to be the main driver of juvenile performance after experiencing small salinity differences during the larval stage. If carryover effects from the larval oysters' environmental experience were driving juvenile performance regardless of the environmental conditions, the physiological response of juvenile oysters would differ based on their larval experience only, and there would be random loss of alleles in larval and juvenile oysters due to bottleneck effects of metamorphosis-associated mortality events, and no large shifts in allele frequencies (Fig. 2a). If phenotypic plasticity were driving juvenile performance, there would be no large shifts in allele frequencies, similar to carryover effects, and physiological responses would be correlated with the juvenile environment rather than the larval

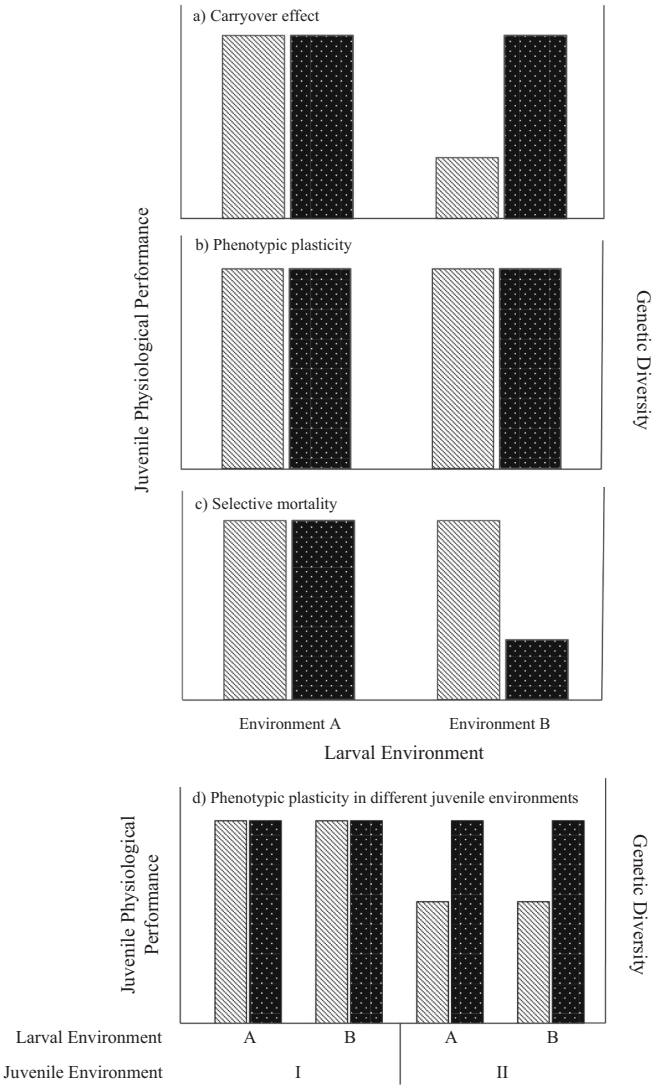

Fig. 2. Hypothesized patterns of physiological performance (gray strips) and non-random loss of genetic diversity (black polka dots) of juvenile oysters in common-garden environmental conditions (a-c) as a function of their larval environment (Environment A, Environment B) and environmental-tolerance mechanism. Carryover effects (a) would not affect the genetic diversity but would decrease physiological responses in juvenile oysters that experienced suboptimal environmental conditions as larvae. Phenotypic plasticity (b) would cause juvenile genetic diversity and physiology to be similar regardless of larval experience. Selective mortality (c) would increase the non-random loss of genetic diversity in oyster larvae experiencing suboptimal conditions and remain low into the juvenile stage with no effect on juvenile physiology. Lastly, if juvenile oysters are placed into different grow-out environments and physiology is driven by phenotypic plasticity (d), juvenile performance may vary between the grow-out environments, not previous larval experience. While binary responses were shown in the graphs above for clarity, it is likely that more than one mechanism may be simultaneously driving organismal performance. In this theoretical example, high physiological performance represents a positive indicator of oyster health.

environmental conditions (Fig. 2b,d). If selective mortality did occur prior to, or during, metamorphosis and was driving juvenile performance, non-random loss of genetic diversity would be expected in juvenile oysters based on their larval experience. Furthermore, the physiological responses of juvenile oysters would be similar regardless of the larval environmental experience due to selection for beneficial genotypes under those environmental conditions (Fig. 2c). It is possible that two, or even all three, mechanisms could simultaneously impact performance, which may yield a range of possible performance outcomes.

Because young, hatchery-reared oysters can be distributed across a number of varied environmental conditions depending on the farm location, the performance of juvenile oysters placed into two different grow-out environments was examined. It was hypothesized that similar juvenile oyster performance between two locations with differing juvenile environments would be evidence for the role of selective mortality or carryover effects stemming from the larval experience (Figs. 2a, c). It is also possible that selective mortality during the juvenile stage itself could produce this pattern (Fig. 2c), which would be informed by survival and changes in genetic diversity of oyster juveniles. If juvenile oyster performance differed between sites, phenotypic plasticity may be more important in shaping juvenile oyster performance in response to their immediate environment (Fig. 2d). Lastly, if larval oysters lack environmental-tolerance mechanisms, including mechanisms beyond those of focus in this study, it was hypothesized that significant mortality would likely occur prior to metamorphosis, a scenario irrelevant to effects on juvenile oyster performance.

2. Materials and methods

2.1. Experimental design

To examine the relative influence of carryover effects and phenotypic plasticity associated with salinity exposure of *C. virginica* larvae, oysters were reared from fertilization through settlement and metamorphosis (i.e., the settlement phase) under different ecologically relevant salinities, thirteen and fifteen. Larvae were assessed for survival, growth, oxidative stress, and genetic diversity at multiple timepoints. Juvenile oysters were then deployed to two experimental field sites in the York and Rappahannock rivers, two tributaries of Chesapeake Bay with active aquaculture production and of known differing salinities (York: 19.57 ± 1.70 [CBNERR-VA VIMS, 2018]; Rappahannock: 15.85 ± 1.99 [M. Congrove, unpublished data]; mean ± standard deviation), to mimic the grow-out phase of oyster aquaculture on farms. After 4 and 16 weeks, oysters were subsampled for physiological and genetic parameters to test for the relative influence of carryover effects and phenotypic plasticity from the larval salinity treatments. Genetic diversity was measured at each experimental stage alongside survival to confirm the hypothesis that any observed differences among treatments and experimental stages were not due to selective mortality, specifically large shifts in allele frequencies, or loss of diversity.

As briefly mentioned above, two salinity treatments were used – thirteen (S_{13}) and fifteen (S_{15}) – whose levels were based on environmental data provided by our commercial hatchery partner, Oyster Seed Holdings, Inc. (OSH), located on the Rappahannock River in Grimstead, VA, and retrieved from the NOAA CBIB Stingray Point buoy (Fig. 1). Average ambient and frequent minimum salinity values that occurred during the hatchery's 2016 and 2017 operating seasons were chosen; due to an anomalously wet spring season in 2018 in Virginia, S_{13} served as the control treatment representative of incoming water salinity at the hatchery. The two treatment groups were cultured at the Virginia Institute of Marine Science (VIMS) in the Aquaculture Breeding and Genetics Technology Center (ABC), receiving manipulated water from the York River (see section *2.1.1 Treatment Water Generation*). To evaluate whether larval culturing at VIMS produced representative results for hatchery production, a third treatment group, referred to as hatchery (S_H), was cultured at OSH, receiving unmanipulated water from the Rappahannock River. To ensure continuity among all treatments, larval culturing practices at OSH were adopted such that all cultures received water changes, food, and sampling on the same days.

2.1.1. Treatment water generation

Water from the York River was mechanically filtered through sand, cartridge, and ultraviolet systems, and finally through 1 μm mesh. After filtration, the water was chemically manipulated to create the S_{13} and S_{15} treatment conditions at VIMS ABC while maintaining similar pH, temperature, and dissolved oxygen conditions. Briefly, salinity was manipulated using additions of deionized water or Instant Ocean®, and then alkalinity was adjusted to match the alkalinity measured from S_H through additions of 1 N NaOH or 1 N HCl, following Lunden et al. (2014; see *S1.1 Treatment Water Generation*). Dissolved oxygen and pH were maintained at atmospheric equilibrium by bubbling room air directly into the culture tanks. The culture tanks were housed within a temperature-controlled room that maintained the water temperature around 25 °C. The S_H treatment group at OSH received 1 μm mechanically filtered water from the Rappahannock River at ambient salinity.

2.2. Larval culturing

Triploid *C. virginica* larvae (two days post-fertilization) were obtained from OSH in Summer 2018. Triploid, rather than diploid, larvae were used since >97% of oyster aquaculture in Virginia utilizes triploid oysters (Hudson, 2019). Female, diploid oysters from broodstock line LOLA ($n = 33$; Bushek and Allen, 1996) and male, tetraploid oysters from broodstock line GNL ($n = 4$; Guévélou and Allen, 2016) were strip-spawned (Allen and Bushek, 1992). Both broodstock lines are produced by VIMS ABC for their disease resistance and are commonly used by commercial hatcheries in Virginia. Mantle and/or gill tissue was subsampled from all adult parents and stored in 95% ethanol for genetic assessment. All eggs were pooled and rinsed to remove harmful bacteria, then divided into four aliquots prior to fertilization. Each aliquot was then fertilized by sperm from one male to generate half-sibling families. After fertilization was completed (~ 1 h), embryos were pooled and rinsed to remove any remaining sperm and bacteria. Embryos were then reared at OSH at a stocking density of 30 embryos mL^{-1} for 48 h under ambient conditions prior to transport to VIMS and the experimental start.

Beginning at 2 days-post-fertilization (dpf), larvae were cultured through metamorphosis (about 4 weeks) under the three salinity treatments (S_{13}, S_{15}, S_H) using three tanks per treatment to create biological pseudoreplicates. Culturing tanks at VIMS ABC (S_{13} and S_{15}) were 50 L, while those at OSH (S_H) were 100 L. To control for the tank size differences, larval density was standardized across treatments. Larval densities started at 15 larvae mL^{-1} and were gradually reduced to 2 larvae mL^{-1} before metamorphosis following hatchery best practices (M. Congrove, pers. comm.; Table S1). To ensure continuity among the treatments at VIMS ABC and OSH, larvae were fed daily on an ad libitum diet of a mixture of live microalgal species (*Isochrysis galbana*, *Thalassiosira weissflogii*, and *Tetraselmis* sp.) cultured at OSH. To characterize tank conditions during the larval and settlement stages, temperature, dissolved oxygen, pH, conductivity, and salinity were measured with a YSI Pro Plus twice daily in S_{13} and S_{15} treatments and three times per week in S_H. Water samples were collected for measurements of pH and total alkalinity on water change days (see *S1.5 Carbonate Chemistry Analysis*).

A full water change of each larval tank was conducted three times per week to remove any build-up of nitrogenous waste in the culture water. Larval sampling occurred at the water change. Tanks were emptied onto a mesh sieve of a size appropriate for the smallest larvae observed at last measurement to conservatively capture all individuals (Table S1). Larvae were then returned to the tank or concentrated into a small

volume of treatment water for sampling (≤ 1 L). Physiological sample collection occurred at 4, 8, 13, and 18 dpf for larvae in S_{13} and S_{15}. The S_H larvae at OSH were sampled twice, half-way through the larval stage at 8 dpf and before settlement at 18 dpf for comparison with S_{13} control larvae.

At each physiological sampling timepoint, larval abundance was compared to the previous sampling timepoint to calculate percent survival. Larval abundance was determined from a minimum of three sequential counts of 20–50 μL of the larval concentrate. Larvae were then volumetrically sampled for growth (n ≅ 200 individuals), total protein content (n ≅ 5000), and total antioxidant capacity (n ≅ 10,000). Once all samples were collected, larvae were returned to the culturing tank at the appropriate density (Table S1), and samples were preserved at -80 °C until later analysis.

Once larvae had shell lengths (measured parallel to the hinge) larger than 300 μm and showed pre-metamorphic attributes, like eyespots and foot muscle protrusion (termed eyed larvae), approximately 75,000 eyed larvae were harvested per treatment replicate and put into a downwelling set-up with the same larval treatment conditions for the settlement phase (see *S1.3 Settlement*). Prior to settlement, whole oysters from each treatment replicate (n ≅ 500 individuals) were preserved in 95% ethanol for genetic analysis. The settlement phase proceeded under treatment conditions for about a week and a half. Once adult morphological features (e.g., gills) were observed, little to no signs of pre-metamorphic features remained, and shell lengths of individuals were > 700 μm, oysters were placed in common garden conditions in the ABC nursery. During common garden, oysters from all treatments received unfiltered York River water in the same upwelling system for about 1.5 months (51 days) until they were large enough for field deployment (> 5 mm; see *S1.4 Common garden)*.

2.3. Juvenile field deployment

Oysters from all treatment replicates (n = 2000 individuals) were deployed to two experimental field sites in the Rappahannock (RR) and York (YR) rivers, tributaries of Chesapeake Bay. Prior to the deployment of oysters, the initial shell length of 50 randomly selected individuals from each treatment replicate was measured.

Oysters were deployed in mesh bags inside single-tiered cages placed at the sediment surface in the intertidal zone with similar estimated levels of tidal exposure. Environmental data were collected at each site using a SeapHOx™ (V1 and V2, Sea-Bird Scientific) that recorded pH, temperature, salinity, and dissolved oxygen every 10 min. Carbonate chemistry samples were collected every two weeks for field validation of the SeapHOx™ (see *S1.5 Carbonate Chemistry Analysis*). Data from the Virginia Estuarine and Coastal Observing System were used to fill gaps in the SeapHOx™ time series from the YR location (CBNERR-VA VIMS, 2018). The offset between discrete water samples and SeapHOx pH was calculated for each instrument (RR: 0.14 ± 0.05, YR: 0.13 ± 0.07; mean ± standard error).

At 4 and 16 weeks, oysters were sampled at each site for shell length, survival, total antioxidant capacity, dry tissue weight, and condition index. Cumulative survival was measured using a random sub-sample of 300 individuals (~15% of the total abundance) from each replicate and calculated based on current oyster abundance in each bag and previous sampling without replacement. Live oysters were measured for growth and sampled for physiological measurements of total antioxidant capacity (n = 3 per treatment replicate, whole-body tissue), dry tissue weight (n = 10 per treatment replicate, whole-body tissue), and condition index (n = 10 per treatment replicate, whole individual; see *2.4 Physiological Assays*). Physiological samples were immediately put on dry ice and later stored at -80 °C until analysis. Genetic samples were taken after 16 weeks. 50 randomly selected oysters from each bag were transported on ice to VIMS and measured for size, and a subsample of soft tissue was preserved in 95% ethanol for later genetic workup.

2.4. Physiological assays

As changes in tissue weight can be indicative of phenotypic plasticity (Méthé et al., 2015b), the total protein content of oysters was measured as a proxy for tissue biomass. Total protein content of larval oysters was measured spectrophotometrically using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, 23225; Smith et al., 1985) and a SpectraMax® iD3 (Molecular Devices) microplate reader. Briefly, oyster samples were homogenized in Milli-Q ultrapure water (Millipore Sigma) and incubated with the working reagent at 37 °C for 30 min before the absorbance at 562 nm was measured at room temperature. Each sample from the treatment replicates was measured in triplicate. Total protein content in the oyster samples were calculated using a standard curve of known concentrations of bovine serum albumin (0–2000 μg mL^{-1}).

Shifts in cellular stress molecules can indicate the response of organisms to changes in their environment (Seebacher et al., 2015), total antioxidant capacity was measured as a proxy for oxidative stress. Total antioxidant capacity was measured using the ferric reducing/antioxidant potential (FRAP) assay (Griffin and Bhagooli, 2004). First, tissue samples were homogenized in 10 mM Tris-HCl buffer. Homogenized samples were then mixed with a working reagent (300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-*s*-triazine, and 20 mM $FeCl_3 \cdot 6H_2O$) followed by a 6-min incubation at 37 °C. Each sample from the treatment replicates was measured in triplicate. The oxidizing reaction produces a colored compound whose absorbance can then be measured at 595 nm using the SpectraMax® iD3 microplate reader at 37 °C. FRAP in oyster tissues was calculated using a standard curve of known oxidized Fe^{II}-concentrations using 20 mM $FeCl_3 \cdot 6H_2O$ (0–1000 μM), and then normalized to total protein content to reduce bias from potential size differences across treatments.

Similar to tissue weight and total protein, changes in shell length can be indicative of phenotypic plasticity (Méthé et al., 2015b). To measure the shell length of larval and early juvenile oysters, a minimum of 100 randomly selected individuals were photographed using a compound microscope (Olympus BX51) or dissection microscope (Nikon SMZ1500), respectively. ImageJ (Schneider et al., 2012) was then used to measure area and longest length (hinge to bill) of 50 randomly selected oysters. Calipers were used to measure the shell length of juvenile oysters during the field deployment.

Juvenile dry tissue weight was measured to indicate a change in weight after field deployment began. In addition, condition index was measured to assess the general health of the juvenile oysters (Rainer and Mann, 1992). Intact oysters were gently cleaned with a toothbrush under DI water to remove any sediment or other material and then shucked. Tissue and shells were dried individually at 60 °C until a constant weight was achieved (± 0.001 g; ~ 2 weeks). Condition index was calculated as a ratio of the dry weight of whole-body tissue to dry weight of the shell (Rainer and Mann, 1992).

2.4.1. Physiological statistical analyses

Linear mixed effects models were used to estimate any treatment effects on individual water conditions (temperature, salinity, dissolved oxygen, pH, total alkalinity, dissolved inorganic carbon, and aragonite saturation state) during larval and settlement stages, with larval salinity treatment as a fixed factor and tank replicate as a random factor (*nlme* package in the statistical program R, version 3.1–155; [Pinheiro et al., 2020; Pinheiro and Bates, 2000]). All treatment water parameters satisfied the assumptions for parametric testing.

ANCOVAs were used to estimate direct effects of salinity conditions on individual larval oyster traits (shell growth, total protein content, total antioxidant capacity, and survival), with larval treatment as the fixed effect, age as a continuous cofactor, and tank replicate as a random factor (*nlme* package in R). An ANOVA was used to estimate treatment effects on the shell length of eyed larvae was analyzed because age was not needed as a cofactor. Total protein content was log transformed and

shell growth was square root transformed to meet assumptions of normality and homogeneity. All other larval oyster traits met the necessary statistical assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was conducted using *stats* (R Core Team, 2022), and the Levine's test was conducted using *car* (Fox and Weisberg, 2019).

A Kruskal-Wallis test was conducted to compare water conditions (temperature, salinity, dissolved oxygen, pH, total alkalinity, dissolved inorganic carbon, and calcite saturation state) from the juvenile field deployment between field locations of the juvenile deployment (*stats*; R Core Team, 2022) because parameters did not meet the assumptions of normality and homogeneity of the Shapiro-Wilkes and Levine's tests, respectively.

An independent two-sample student's *t*-test was conducted to assess initial signs of carryover effects or phenotypic plasticity associated with salinity treatments using oyster traits measured after the completion of the settlement phase (shell length, total protein content, and total antioxidant capacity) (*stats*; R Core Team, 2022). Normality and homogeneity assumptions of the Shapiro-Wilkes and Levine's tests, respectively, were met (Fox and Weisberg, 2019; R Core Team, 2022).

To further observe potential carryover effects of larval salinity experience or phenotypic plasticity, as well as shifts in relative influence of carryover effects in different environmental conditions, ANCOVAs were conducted for individual juvenile oyster traits (shell growth, dry tissue weight, total antioxidant capacity, condition index and survival), with larval salinity treatments and experimental field sites as fixed effects and time as a continuous cofactor (*nlme* package in R). Dry tissue weight was natural log transformed to meet assumptions of normality and homogeneity. All other juvenile oyster traits met the necessary statistical assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity (Fox and Weisberg, 2019; R Core Team, 2022). All statistical analyses were completed in R (version 4.1.3; [R Core Team, 2022]).

2.5. Genetic analysis

2.5.1. DNA isolation

It was empirically determined that DNA could be consistently isolated and amplified from individual larval oysters of 300 μm size and larger (data not included). Thus, only eyed larvae (18 dpf) and juvenile oysters (16 weeks after field deployment) were used for genetic analysis, in addition to broodstock samples, which were used to establish baseline genetic diversity. Oysters were randomly selected at the beginning of the settlement phase as eyed larvae (hereafter referred to S_{eyed}) and at the end of the field deployment as juveniles from all treatment replicates. All tissue samples from broodstock, S_{eyed}, and juvenile oysters underwent a DNA extraction protocol using Puramag™ carboxylated magnetic beads (McLab, San Francisco, CA; See *S1.6 Genetics*). DNA quantity was assessed with a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific), and isolates were diluted, if necessary, to 50 ng μL^{-1} or less prior to amplification.

2.5.2. Microsatellite data acquisition

To evaluate loss of genetic diversity, *C. virginica* primers targeting tri- and tetranucleotide microsatellite loci were tested, and amplification conditions were optimized for oysters from Virginia (Brown et al., 2000; Carlsson and Reece, 2007; Reece et al., 2004; Wang et al., 2009; Wang and Guo, 2007; see *S1.6 Genetics* for optimization methods). A total of 18 microsatellite loci were optimized into five multiplex groups (Table S2). Broodstock, S_{eyed}, and juvenile DNA isolates underwent multiplex PCR amplification using the Qiagen Type-It Microsatellite PCR Kit (Qiagen) with the following components: 3.25 μL Type-It Mastermix, 0.5 μL 1 mg mL^{-1} BSA, 0.625 μL primer mix (consisting of 1 part locus specific forward primer modified to include a universal tail sequence, e.g., T3, to 4 parts locus specific reverse primer to 4 parts of the fluorescently labeled universal primer), 1.125 μL PCR water, and 0.5 μL template DNA. The PCR cycling protocol was as follows: 95 °C initial denaturation for 5 min, 28 cycles of 95 °C denaturation for 30 s, 90 s at the annealing temperature, 72 °C extension for 30 s, and a final extension at 60 °C for 30 min. Alleles were size separated on either an ABI Genetic Analyzer 3130XL (broodstock and juveniles) or 3500 (S_{eyed}) with a GeneScan™ 600 LIZ® Size Standard v2.0 (ThermoFisher Scientific). Broodstock chromatographs were used to size alleles in GeneMarker V2.6.0 (Softgenetics, State College, PA) and establish allele panels for each locus. S_{eyed} and juvenile alleles were then sized based on the broodstock panels. All alleles were sized twice, and where a consensus could not be reached, data was coded as missing.

2.5.3. Genetic statistical analyses

Alleles at microsatellite loci that were not able to be scored consistently, or that were present in offspring but not detected in the broodstock genotypes, were excluded from further analyses. Any individual oyster or microsatellite locus with $>$ 25% missing data was excluded from further analyses. The remaining data were converted to a Genepop file format (Raymond and Rousset, 1995; Rousset, 2008). Oysters were grouped by sampling event (broodstock, S_{eyed}, juveniles), treatment (S_{13}, S_{15}), and field deployment location (YR, RR), with each unique combination of these three factors constituting a sample. Data from all three treatment replicates were merged for each sampling timepoint. To correct for unknown dosage in the triploid and tetraploid oysters (e.g., AAB vs. ABB for triploids), which makes accurate estimation of allele frequencies difficult, the dosage correction tool implemented in GenoDive v3.0 (Meirmans, 2020) was used to impute all missing data based on sample-by-sample allele frequencies. Genetic diversity indices including number of alleles, allele frequencies, and observed (H_O) and expected heterozygosity (H_S), were calculated for each sample in GenoDive (Meirmans, 2020). GenoDive was also used to compute pairwise F_{ST} values, and significance was assessed based on 9999 iterations of the data using an initial $\alpha = 0.05$. *P*-values were corrected for the false-discovery rate (FDR; [Benjamini and Hochberg, 1995]). The Genepop software (Raymond and Rousset, 1995; Rousset, 2008) was used to identify significant differences in genic (allelic) frequencies among samples on a locus-by-locus basis using the exact G test, and significance was assessed with 100 batches of 1000 Markov Chain Monte Carlo (MCMC) iterations each following a burn-in of 1000 iterations. G-tests implemented in Genepop were used to identify significant differences among samples based on allele frequencies, and significant locus-by-locus comparisons ($<$0.05) were summed for each pairwise comparison. To identify samples with rare alleles, a private alleles analysis was completed in Genepop.

3. Results

3.1. Water conditions

3.1.1. Larval, settlement, and nursery phases

Average larval culture water conditions were significantly different between laboratory treatment groups for many parameters measured (Tables 1, S3). Salinity differed by 1.77 units, dissolved oxygen differed by 0.2 mg L^{-1}, total alkalinity differed by 29 μmol kg^{-1}, and dissolved inorganic carbon differed by 35 μmol kg^{-1} (Table 1). All other water characteristics were not statistically different between treatments. Due to the anomalously wet spring and lack of control over culture conditions at OSH, water conditions tended to differ between S_{13} and S_H (Table 1). Specifically, salinity was lower, while temperature, pH, and aragonite saturation state were all higher in the S_H treatment than in S_{13} (Table 1).

During the settlement phase, except for salinity ($S_{13} = 13.20 \pm 0.02$, $S_{15} = 15.00 \pm 0.02$; mean $\pm$ standard error) and dissolved oxygen ($S_{13} = 7.28 \pm 0.07$ mg L^{-1}, $S_{15} = 7.09 \pm 0.05$ mg L^{-1}; mean $\pm$ standard error), average water conditions were not significantly different between laboratory treatment groups (Tables S4, S5). Oysters experienced

Table 1
Average water chemistry conditions experienced by larvae in salinity treatment groups. Values represent mean ± standard error calculated from treatment replicates over the entire larval period. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), and $\Omega_{aragonite}$ (saturation state of aragonite) are calculated values. Asterisks (*) indicate significant differences between water properties of laboratory treatments ($p < 0.05$; linear mixed effects model; Table S2).

	Treatments		
	Laboratory		
Water Properties	Thirteen (S_{13})	Fifteen (S_{15})	Hatchery (S_H)
Larval Culture			
Temperature (°C)	25.20 ± 0.08	25.07 ± 0.09	27.41 ± 0.17
Salinity	13.06 ± 0.01*	14.83 ± 0.02*	11.80 ± 0.05
Dissolved Oxygen (mg L^{-1})	7.03 ± 0.05*	6.83 ± 0.05*	6.70 ± 0.07
pH_{tot}	7.56 ± 0.01	7.51 ± 0.02	7.74 ± 0.01
Total Alkalinity (μmol kg^{-1})	1418 ± 4*	1447 ± <1*	1446 ± <1
DIC (μmol kg^{-1})	1413 ± 5*	1448 ± 3*	1412 ± 1
$\Omega_{aragonite}$	0.51 ± 0.01	0.49 ± 0.02	0.76 ± 0.01

temperatures of ~25 °C, pH values of ~7.8, total alkalinities of ~1510 μmol kg^{-1}, and calcite saturation states of ~1.25 during the settlement phase (Table S4). After the settlement phase was completed, oysters experienced temperatures of ~28 °C, salinities of ~15.2, pH values of ~7.6, total alkalinities of ~1410 μmol kg^{-1}, and calcite saturation states of ~1.07 in common garden during the nursery phase (Table S6).

3.1.2. Juvenile field deployment

Oysters experienced significantly different water conditions between the two field sites over the deployment period (Table 2, S7). On average, salinity was higher by 5.29 units, pH was lower by 0.17 units, total alkalinity was greater by 184 μmol kg^{-1}, and dissolved inorganic carbon was greater by 188 μmol kg^{-1} in the YR than in the RR. Though not significantly different, temperature was greater by 0.95 °C on average in the YR. Calcite saturation state and dissolved oxygen were similar between the two locations.

3.2. Comparisons of allele frequencies based on the larval environment

Three loci and 35 individuals were dropped from the final dataset due to missing data, resulting in a final dataset of 15 loci, 37 broodstock individuals, 112 eyed larvae, and 183 juveniles. Broodstock genotypes consisted of 102 alleles across the 15 microsatellite loci (Table S8). Alleles per locus ranged from three (Cvi2k14, RUCV80, RUCV109, RUCV159) to 15 (Cvi13). A private alleles analysis revealed five private alleles across five different loci, all of which were unique to the broodstock. Overall, 21 and 18 broodstock alleles were not recovered in the first post-fertilization analysis of the offspring ($S_{13\text{-eyed}}$ and $S_{15\text{-eyed}}$, respectively), though many of these alleles were detected in juvenile samples at the end of the experiment at a lower frequency relative to the broodstock (Table 3). The number of broodstock alleles recovered when samples were separated by life stage and salinity treatment ranged from 81 to 88, and when salinity treatments were combined across sampling events, the number of alleles ranged from 90 to 94 (Table 3).

Observed and expected heterozygosity (H_O and H_S, respectively) for the microsatellite data for all samples after dosage correction are displayed in Table 3. Observed heterozygosity (H_o) values were lower than expected heterozygosity (H_s) values in all samples. The highest number of significant locus-by-locus genic differences (9 of 11 significant differences) and the highest F_{ST} values (0.033–0.047; Table S9) involved comparisons between the broodstock and all offspring samples. Genic differences across all 15 loci were significant between all sample pairs except RR_{13} and YR_{15}, with a p-value of 0.070.

Pairwise F_{ST} values between S_{eyed} treatments and the corresponding juvenile samples were all statistically significant with F_{ST} values around 0.01 and 4–6/15 significant genic (locus) differences (Table 4, S10). Pairwise F_{ST} comparisons of juvenile field deployment location by salinity treatment groups resulted in estimates that were extremely small but were in most cases statistically significant, except for the comparisons RR_{15} vs. YR_{13} and RR_{13} vs. YR_{15} (Table 5). All cross-treatment comparisons produced F_{ST} values of <0.01 and between 2/15 and 4/15 significant genic differences.

Within salinity treatments, juvenile samples placed in the two field locations (RR and YR) retained similar numbers of alleles; 88 of the original 102 alleles were detected in both S_{13} juvenile samples at the end of the experiment. For the S_{15} samples, 81 (RR) and 85 (YR) alleles were detected (Tables 3, S11). The pairwise comparison of RR_{13} and YR_{13} was statistically significant, as was the comparison of RR_{15} and YR_{15} and the number of significant genic differences were 3/15 and 5/15, respectively (Table 5). F_{ST} values and counts of significant genic differences were on a similar level to cross-treatment comparisons within the same field sites (Table S9).

3.3. Direct and carryover effects of larval environment on oyster physiology

In general, larval cultures raised under ecologically relevant salinities (Fig. 1) were physiologically similar, with no direct effects of

Table 2
Average water chemistry conditions during the field deployment period for juvenile oysters. Field deployment conditions are represented by mean ± standard error calculated over the entire field deployment period (four months) in the York and Rappahannock Rivers. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon) and $\Omega_{calcite}$ (saturation state of calcite) are calculated values. Asterisks (*) indicate significant differences between water properties of field sites ($p < 0.05$; Kruskal-Wallis; Table S2).

	Field deployment	
	York River (YR)	Rappahannock River (RR)
Temperature (°C)	18.48 ± 0.07	17.53 ± 0.08
Salinity	14.43 ± 0.01*	9.14 ± 0.01*
Dissolved Oxygen (mg L^{-1})	6.52 ± 0.01	6.31 ± 0.01
pH_{tot}	7.74 ± 0.01*	7.91 ± 0.01*
Total Alkalinity (μmol kg^{-1})	1491 ± 1*	1307 ± 1*
DIC (μmol kg^{-1})	1464 ± 1*	1276 ± 1*
$\Omega_{calcite}$	1.11 ± 0.01	1.12 ± 0.01

Table 3
Indices of genetic diversity, including sample size (n), total alleles, observed heterozygosity (H_O), and expected heterozygosity (H_S) calculated from 15 microsatellite loci with dosage correction. Results are listed for each individual sample (sampling event by treatment), each sampling event (pre-metamorphosis (S_{eyed}), and Rappahannock River (RR) and York River (YR) juvenile outplant locations), each salinity treatment (salinity of 13 (S_{13}) and salinity of 15 (S_{15})), and for all samples together (Total; with 95% confidence intervals).

Population	n	Total Alleles	H_O	H_S
Sampling event by larval treatment				
$S_{13\text{-eyed}}$	53	81	0.445	0.558
$S_{15\text{-eyed}}$	59	84	0.464	0.556
RR_{13}	45	88	0.467	0.557
RR_{15}	47	81	0.475	0.555
YR_{13}	48	88	0.458	0.552
YR_{15}	43	85	0.453	0.540
Sampling event				
S_{eyed}	112	90	0.431	0.549
RR	92	91	0.454	0.573
YR	91	94	0.440	0.561
Larval treatment				
S_{13}	146	94	0.456	0.559
S_{15}	149	95	0.464	0.554
Total	295	102	0.442	0.561
CI 2.5%	–	–	0.353	0.460
CI 97.5%	–	–	0.523	0.659

Table 4
Pairwise comparisons with F_{ST}'s calculated from 15 microsatellite loci are included between pre-metamorphic larvae (S_{eyed}) and juveniles within the same salinity treatments deployed in either the Rappahannock River (RR) or York River (YR). Comparisons with significant FDR-corrected p-values are bolded and corresponding p-value is provided in parentheses. The sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci are indicated. Subscripts indicate the larval salinity treatment (13 or 15).

	F_{ST} (p-value)	Genic Differentiation
$S_{13\text{-eyed}}$ - RR_{13}	**0.008** (0.001)	5
$S_{13\text{-eyed}}$ - YR_{13}	**0.012** (0.001)	6
$S_{15\text{-eyed}}$ – RR_{15}	**0.010** (0.001)	6
$S_{15\text{-eyed}}$ - YR_{15}	**0.007** (0.001)	4

Table 5
Pairwise comparisons between salinity treatments in juvenile samples, with F_{ST}'s calculated from 15 microsatellite loci on the upper diagonal, with comparisons with significant FDR-corrected p-values bolded and corresponding *p*-value is provided in parentheses, and the sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci on the lower diagonal. Samples are juveniles deployed in either the Rappahannock River (RR) or York River (YR), with the subscript indicating the salinity treatment (13 or 15).

	RR_{13}	YR_{13}	RR_{15}	YR_{15}
RR_{13}	–	**0.010** (0.001)	**0.006** (0.003)	0.002 (0.087)
YR_{13}	3	–	0.001 (0.313)	**0.013** (0.001)
RR_{15}	2	3	–	**0.010** (0.001)
YR_{15}	4	4	5	–

salinity detected. Shell length, total protein content, and total antioxidant capacity did not differ between larvae reared in the S_{13} and S_{15} treatments (Fig. 3, Table 6). Survival remained above 75% throughout larval development and did not vary significantly by salinity treatment or larval age (Table 6). Larval age had a significant effect on shell length, total protein content, and total antioxidant capacity (Table 6). Both shell length and total protein content increased from 4 to 18 dpf by 220% and 561%, respectively (Fig. 3a-b). Total antioxidant capacity decreased 62% in larval oysters between 4 and 18 dpf (Fig. 3c). Prior to settlement, the shell length of eyed larvae was also similar between treatments (Fig. S2, Table 6). When evaluating if the results from oysters reared at VIMS were representative of hatchery production, the hatchery comparison treatment (S_H) tended to differ physiologically from S_{13} even though salinities were relatively similar (Fig. S1).

Larval salinity did not directly affect oysters during the settlement phase. After the settlement phase was completed, there were no significant differences in shell length, total protein content, and total antioxidant capacity between treatment groups (Fig. S3, Table S12).

Throughout the juvenile field deployment when carryover effects were assessed, juvenile physiology was similar among larval treatment groups (Table 6). Specifically, survival, shell length, dry tissue weight, total antioxidant capacity, and condition index did not significantly differ based on what salinity oysters experienced as larvae (Table 6).

3.4. Phenotypic plasticity in response to juvenile environments

During the juvenile stage when oysters were deployed in the York and Rappahannock Rivers, physiology of the oysters differed significantly by site and time (Table 6). Specifically, condition index, dry tissue weight, and shell length all changed significantly over time, increasing by 11%, 102%, and 20%, respectively, over four months while oysters were deployed in the York and Rappahannock Rivers (Fig. 4b,c,e). RR oysters had greater survival (13% difference), shell length (14% difference), condition index (38% difference), and dry tissue weight (78% difference) than the YR oysters at the end of deployment (Fig. 4a-d). RR oysters also had lower total antioxidant capacity than YR oysters by 20% (Fig. 4e).

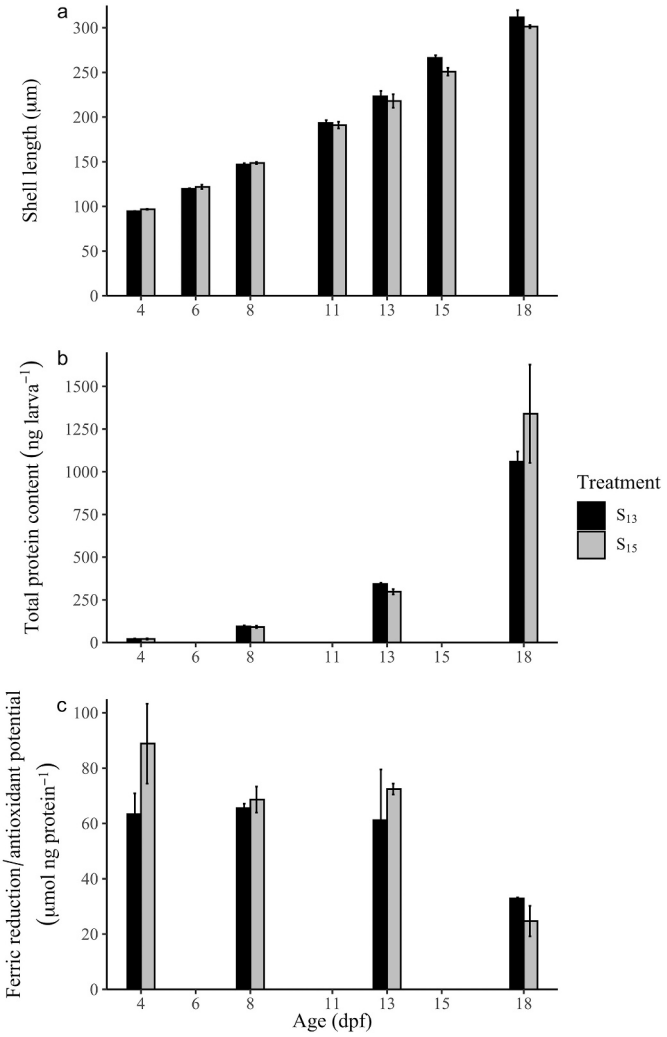

Fig. 3. Physiology of oyster larvae cultured under salinities of thirteen (S_{13}; black) and fifteen (S_{15}; gray). Larvae began to show signs of metamorphosis at 18 days post fertilization. Shell length (a), measured perpendicular from hinge to edge of larval shell, total protein content (b), and total antioxidant concentration (c) were similar among larvae raised at the different salinities. Bars represent mean ± standard error.

4. Discussion

Oysters encounter different water conditions during hatchery and grow-out phases of aquaculture that could impact performance. Specifically, varied larval environmental experiences could affect the relative importance of various environmental-tolerance mechanisms in shaping the performance of eastern oysters, *C. virginica*, through the hatchery-to-farm transition in oyster aquaculture. As hatchery-reared oysters are distributed to farms with differing environmental conditions, it is possible that the larval experience could drive juvenile performance through selective mortality (Fig. 2c) or carryover effects (Fig. 2a). If juvenile oyster performance is more so shaped by their immediate environmental conditions, then phenotypic plasticity may be driving juvenile performance (Fig. 2b,d). In the present study, phenotypic plasticity likely played a more prominent role than carryover effects in shaping juvenile *C. virginica* performance after individuals experienced small differences in hatchery salinity conditions ($\leq$ 2 units). Juvenile oyster physiology varied with field deployment location (i.e., varied water conditions), not with previous larval experience based on a small salinity difference of two (Fig. 4, Table 6). Genetic diversity also remained relatively similar throughout the study, with no large shifts in

Table 6
Results of mixed effects linear models, one model per life stage, used to individually compare larval and juvenile physiological metrics. Age and time were included as covariates in models of larval and juvenile metrics, respectively. Tank was included as a random factor for larval comparisons. Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest.

	Statistical Value	*P* value
Larval Stage		
Survival		
Age	$F_{(1,39)} = 1.057$	0.310
Treatment	$F_{(1,39)} = 0.764$	0.388
Larval Shell Length		
Age	$F_{(1,39)} = 4945$	<0.001*
Treatment	$F_{(1,39)} = 2.232$	0.143
Eyed Larval Shell Length		
Eyed Stage	$F_{(1,8)} = 266$	<0.001*
Treatment	$F_{(1,8)} = 1.457$	0.262
Total Protein Content		
Age	$F_{(1,21)} = 826$	<0.001*
Treatment	$F_{(1,21)} = < 0.001$	0.988
Total Antioxidant Capacity		
Age	$F_{(1,21)} = 19.80$	<0.001*
Treatment	$F_{(1,21)} = 1.199$	0.287
Juvenile Stage		
Survival		
Time	$F_{(1,20)} = 0.631$	0.436
Site	$F_{(1,20)} = 51.76$	<0.001*
Treatment	$F_{(1,20)} = 0.046$	0.833
Condition Index		
Time	$F_{(1,20)} = 4.908$	0.039*
Site	$F_{(1,20)} = 53.30$	<0.001*
Treatment	$F_{(1,20)} = 0.680$	0.419
Shell Length		
Time	$F_{(1,20)} = 29.02$	<0.001*
Site	$F_{(1,20)} = 17.94$	<0.001*
Treatment	$F_{(1,20)} = 3.606$	0.072
Dry Tissue Weight		
Time	$F_{(1,20)} = 29.78$	<0.001*
Site	$F_{(1,20)} = 22.51$	0.001*
Treatment	$F_{(1,20)} = 3.856$	0.064
Total Antioxidant Capacity		
Time	$F_{(1,20)} = 0.482$	0.495
Site	$F_{(1,20)} = 8.134$	0.009*
Treatment	$F_{(1,20)} = 0.439$	0.515

allelic frequencies (Tables 3–5). Conserving physiological function along with genetic diversity across various life stages is likely important to the success of oyster performance in dynamic coastal environments, a finding supported by the results of previous studies (Jones et al., 2019; Modak et al., 2021; Sirovy et al., 2021).

Phenotypic plasticity likely shaped oyster performance during the larval stage in the hatchery and in juveniles during field deployment. Oyster physiology was similar between treatment groups (S_{13}, S_{15}) during the larval stage and within sites at deployment where oysters experienced different water conditions to those in the hatchery (Tables 1, 2). Allelic differences were also highly random within each treatment (Fig. 2). Plasticity is a known mechanism of environmental tolerance utilized by organisms living within highly variable environments, such as eastern oysters (Hofmann and Todgham, 2010; Seebacher et al., 2015). Other studies confirm eastern oysters maintain high plasticity in response to various environments and conditions (Chapman et al., 2011; Eierman and Hare, 2016). One characteristic of highly plastic organisms, and a general criterion for identifying plasticity (Barbosa et al., 2022), is their ability to respond relatively quickly to changes in environmental conditions, which was observed in juvenile oysters within weeks of field deployment under different environments (present study) and in transplant experiments with adult oysters (Eierman and Hare, 2016; Sirovy et al., 2021).

Small salinity differences between larval cohorts likely do not impact oyster performance after the larval stage, as we detected no evidence of carryover effects from the larval salinity experience after the settlement phase (i.e., time inclusive of both settlement and metamorphic processes) or during field deployment. It is possible that carryover effects were starting to emerge by 16 weeks in the York River (Fig. 4b), which could be further explored with a more detailed time course. Within experimental field deployment locations in the Rappahannock and York Rivers, juvenile oysters performed similarly with respect to physiological measurements of survival, shell length, condition index, dry tissue weight, and total antioxidant capacity regardless of their larval experience (Fig. 4, Table 6). The small difference in salinity between treatments (2 units) may not have directly produced any physiological differences in the larvae; alternatively, the larvae may have acclimatized to the salinity treatments, removing any initial physiological divergence. Juvenile oysters whose larvae were reared at the commercial hatchery (S_H) were physiologically similar to individuals in the S_{13} treatment during field deployment, despite differences in hatchery conditions (i.e., temperature and pH) at the larval stage. S_H larvae had less total protein content and total antioxidant capacity than those in the S_{13} treatment prior to the settlement phase (Fig. S1), likely due to issues with feeding and other uncontrollable environmental differences with hatchery conditions. By the start of the field deployment within each location, the individuals raised in the S_H treatment were physiologically similar to individuals reared in not only S_{13}, but also S_{15} (unpublished data). The lack of physiological differences among larval treatments at the juvenile stage likely indicates that carryover effects are not a main driver of juvenile performance when larval oysters experience small differences in salinity in the hatchery.

Hatchery culturing conditions alongside small salinity differences likely did not affect the genetic makeup of the animals used in this study beyond the initial spawning event. Allelic diversity declined significantly from the broodstock to offspring (Tables S9 and S11), which has been documented previously in both *C. gigas* and *C. virginica* (Appleyard and Ward, 2006; Hughes et al., 2019; Varney and Wilbur, 2020). On average, survival in oyster culturing after the settlement period is around 10–20% (VIMS ABC, pers. comm.; Ernande et al., 2003; Plough and Hedgecock, 2011), and selective mortality occurring prior to, or during, metamorphosis has been linked to deleterious alleles (Plough, 2018; Plough and Hedgecock, 2011). There was no evidence of differential survival or large shifts in allele frequencies among juvenile oysters after the settlement or field deployment phases; therefore, it is unlikely that selection events related to previous environmental experience occurred (Tables 3–6), potentially due to the small differences in larval salinity being within the optimal range of 10–17 for eastern oysters (McFarland et al., 2022; Scharping et al., 2019). Percent survival after the settlement phase in the current study was consistent with survival in previous studies (~ 30%) with no obvious change in allelic diversity that would support the occurrence of selective mortality since allelic differentiation values remained similar to those prior to settlement (Table S9). In addition, survival was similar between larval salinity treatments throughout the study (Table 6), including individuals within the S_H treatment, further suggesting that survival was likely not genotype-dependent (Tables 3–5, S10, S11).

If juvenile oyster performance was influenced by selective mortality, detection was beyond the limitations of the methods in the present study. Using a more robust locus set (e.g., genome-wide single-nucleotide polymorphisms) to examine the samples in this study may have revealed evidence of selective mortality that was not observed. Furthermore, the relatively low number of samples and loci assayed in this study likely made it difficult to assess non-random genetic selection. Several rare alleles were found within the broodstock used for this study at frequencies below 3.7% and were not detected during the larval stage. The absence of detection was possibly due to a lack of inheritance or equal genetic contribution from the parents (Table S8), which was supported by the lower observed heterozygosity (H_o) values compared to expected values (H_s) in all samples (Table 3), as is common in oysters (Bernatchez et al., 2019; Reece et al., 2004). Alternatively, rare alleles

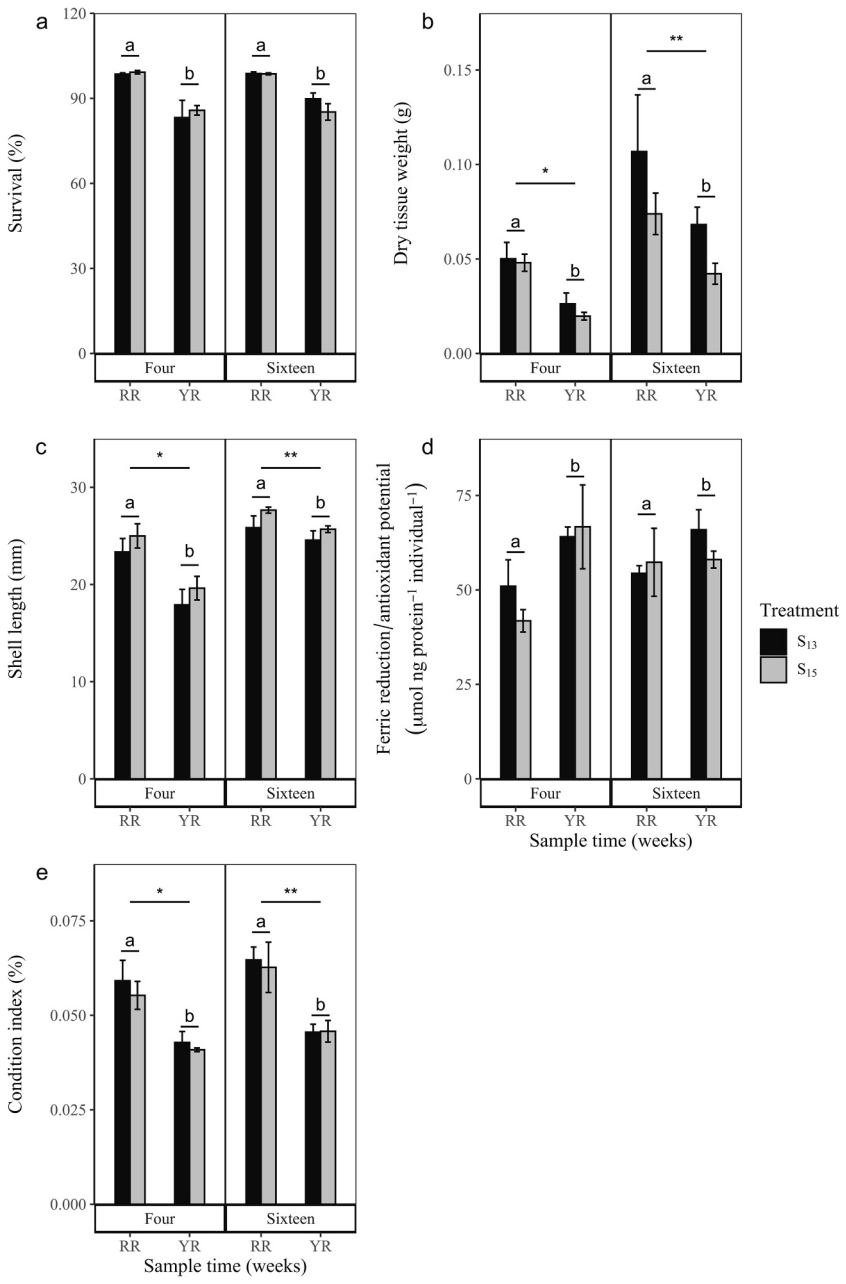

Fig. 4. Effect of in situ environmental conditions and previous larval salinity experience on the physiology of juvenile oysters at four and sixteen weeks. Oysters were deployed in the Rappahannock (RR) and York Rivers (YR) for four months after the common garden period. Overall, the juvenile environment had a significant effect on oyster physiology compared to larval salinity experience. Specifically, RR oysters had increased survival (a), greater dry tissue weight (b), larger shell length (c), measured from hinge to edge of shell, lower total antioxidant capacity (d), and greater condition index (e), measured as the weight of whole-body tissue over the shell, than YR oysters. Oysters that experienced a salinity of thirteen (S_{13}; black) during the larval stage were similar to those raised in a salinity of fifteen (S_{15}; gray) across all physiological metrics and juvenile environmental conditions. Bars represent mean ± standard error. Different letters indicate significant difference between grow-out sites ($p < 0.05$; mixed effects linear model), and asterisks indicate significant difference between sampling weeks ($p < 0.05$; mixed effects linear model).

are more likely to be lost in bottlenecking events (Maruyama and Fuerst, 1985), such as the population reductions that occur during the culturing process (e.g., culture density decreases), or they may remain in the population but not be observed due to the limited random sampling of individuals for genetic analysis. In some cases, broodstock alleles that were not observed during the larval stage were recovered in the juvenile samples, supporting the contention of sampling error. Furthermore, the aquaculture oysters produced in Virginia are mostly triploids, making genetic analysis more complex and the results more difficult to interpret as unknown dosage of biallelic heterozygotes may bias results, and many analyses assume haploid or diploid data and are not optimized for triploids. It is also possible that the genetic diversity of broodstock used was too low such that any selection associated with the experimental design would not likely produce detectable performance differences. Future studies could examine the relative role of selective mortality for broodstock populations with higher genetic diversity.

Carryover effects and selective mortality may play a larger role in shaping oyster performance when environmental differences among larval cohorts or between hatchery and grow-out environments are greater in magnitude than tested in the present study. In previous work on carryover effects, the differences between control and treatment conditions were greater than those used in this study, like a difference of

0.4 pH units on the logarithmic scale (Donelan et al., 2021; Hettinger et al., 2012, 2013; Manuel et al., 2023; Talmage and Gobler, 2011; Torres et al., 2016). Carryover effects of low salinity experience have been found to negatively affect post-metamorphic survival and growth in polychaetes, *Capitella* sp. I (Pechenik, 2006; Pechenik et al., 2001) but benefit juvenile oysters depending on the environmental conditions during the juvenile stage (Manuel et al., 2023). Though several treatment conditions were separated by two to three salinity units, the greatest change from early salinity experience to the later-life stage conditions was twenty salinity units (Manuel et al., 2023; Pechenik et al., 2001). Furthermore, pronounced genetic selection events have been found to occur in oysters under more stressful conditions than implemented in this study (Plough, 2012). With increasing springtime precipitation projected for the mid-Atlantic region under climate change scenarios (Muhling et al., 2018; Walsh et al., 2014), weekly salinity differences may increase in magnitude in the future, potentially impacting larval physiology and genetics between hatchery-reared larval cohorts through carryover effects and selective mortality, an area for future investigation.

Carryover effects and selective mortality may emerge as more important drivers of performance when stressful conditions align with sensitive periods in organismal development. In non-brooding oysters and other bivalve species, the most vulnerable life stage is the larval period, and within that stage, the first 48 h after fertilization is considered the most sensitive and susceptible portion (Barton et al., 2012; Gray et al., 2022). During those first 48 h, larvae are actively precipitating their initial shell, which is energetically expensive and leaves them more exposed to environmental conditions due to its greater surface area (Waldbusser et al., 2013). Furthermore, larvae are utilizing finite, endogenous energy reserves during this time when they are developing their feeding structures (Waldbusser et al., 2015). Our larval exposures started at 48 h post fertilization based on logistics with the commercial hatchery partner, which may have precluded observations of carryover effects. Many studies aiming to observe carryover effects in broadcast spawning oysters and other mollusk species began treatment exposure within hours of fertilization (Clark and Gobler, 2016; Mackenzie et al., 2022; Parker et al., 2015; Shen et al., 2022; Talmage and Gobler, 2009, 2011). Experience of stressful environmental conditions during the first 48 h after fertilization can have negative effects on the rest of the larval stage (Ragg et al., 2019; White et al., 2013). Larvae during the first 48 h post-fertilization may be more sensitive to minute changes in environmental salinities, and therefore small salinity differences like those employed in the current study may induce physiological or genetic differences between larval cohorts if experienced within the first 48 h post-fertilization. An important consideration in future studies is the alignment between the timing of organismal development and exposure to various environmental conditions to better understand potential impacts to marine bivalve populations and aquaculture production.

As no carryover effects from the larval salinity experience were observed, the environmental conditions experienced by juvenile oysters played an important role in their survival and growth. Environmental conditions differed between field deployment locations, potentially influencing juvenile oyster responses, though the present study was not designed to attribute differences in oyster performance to particular in situ water parameters. Salinity, total alkalinity, and dissolved inorganic carbon were all lower at the Rappahannock River site compared to the York River site and correlated with better oyster performance (Fig. 4, Tables 2). Oysters commonly exhibit lower filtration rates with exposure to lower salinities (Casas et al., 2018b; McFarland et al., 2022; Méthé et al., 2015b); however, there is evidence that oysters grown in the Rappahannock River have higher filtration rates compared to those in the York River despite lower average salinities (Mizuta et al., 2021). Though increased filtration rates can be a sign of stress under certain conditions, they can also lead to greater consumption of phytoplankton and particulate organic matter. While chlorophyll-a concentrations and phytoplankton communities were not assessed here, the field site in the Rappahannock River may have had greater algal concentrations or algal diversity than that of the York River during our study period, leading to greater performance of oysters in the Rappahannock River. Similarly, other water conditions that were not measured in the present study, like residence time and currents, which both impact algal concentrations for feeding (Campbell and Hall, 2019), may also have contributed to differences in oyster performance during the juvenile phase.

In the context of shellfish aquaculture operating in mesohaline environments, small salinity differences experienced between larval cohorts after 48 h of fertilization will not likely hinder the production of triploid oyster seed or the performance of the triploid oysters once transitioned to more saline farm environments. Although we know of no evidence that ploidy affects salinity tolerance of oysters with otherwise similar genetic backgrounds, a similar study could confirm the generalizability of our results to diploid oysters. The consensus between this study and previous work that oysters maintain their physiology after experiencing shifts in environmental conditions highlights the potential importance of labile phenotypic plasticity to the performance of these sessile, ectothermic organisms in variable environments. The findings from this study have implications for aquaculture production both in hatcheries with weekly larval cohorts and in the transition from hatchery to farms for grow-out. The important role of phenotypic plasticity is another reason why farm site selection is essential for the performance and success of oyster product since the physiology of juvenile oysters was responsive to their in situ environment and not influenced by larval salinity experiences. Neither carryover effects nor large shifts in allelic frequencies were detected. Other interactions between impacts of in situ environmental conditions on juvenile oyster performance and current hatchery practices for larval rearing could be important for aquaculture production. To further understand the relative importance of phenotypic plasticity, carryover effects, and selective mortality within oyster aquaculture, investigations should focus on identifying whether larval responses to salinity conditions are dependent on other water conditions, like temperature, or exposure to different water conditions within the first 48 h after fertilization. Understanding the link between the hatchery environment and juvenile oyster performance under different farm conditions farms will help improve aquaculture production in the long term, and the approach in the present study is an initial step towards identifying likely mechanisms.

CRediT authorship contribution statement

Annie Schatz: Methodology, Project administration, Investigation, Formal analysis, Validation, Data curation, Writing – original draft, Writing – review & editing. **Jan McDowell:** Conceptualization, Funding acquisition, Resources, Investigation, Validation, Writing – review & editing. **Ellen E. Biesack:** Investigation, Formal analysis, Validation, Data curation, Writing – original draft, Writing – review & editing. **Emily B. Rivest:** Conceptualization, Funding acquisition, Resources, Supervision, Investigation, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work conducted in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740432.

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