Title: Evaluating a short vs. long-term progeny test and investigating physiology associated with survival in extreme low salinity for the eastern oyster *Crassostrea virginica*

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Abstract

Low salinity negatively affects growth and survival in the eastern oyster, which decreases productivity of aquaculture operations along the mid-Atlantic and Gulf Coasts of the United States. With heavy rainfall events predicted to become more frequent, coastal aquaculture operations face increased risk of prolonged exposure to extreme low salinity conditions. While recent experimental work has determined that survival in extreme low salinity (< 3) is a moderately heritable trait in the eastern oyster, further experimental challenges were conducted to investigate the importance of challenge duration and temperature on the survival phenotype and estimation of its genetic parameters to hone the experimental challenge for potential incorporation into a breeding program. Growth (shell height) and algal removal were also assessed to investigate physiological phenotypes associated with differential survival in extreme low salinity. A subset of individuals from 51 half-sibling families were exposed to one of two experimental challenges: a short-term low salinity (2.5) challenge at constant temperature (27°C) for 2 months, or a long-term low salinity (2.5) challenge where temperature was adjusted daily to match local ambient conditions for 6-months. Differential mortality was observed across families for both the short-term and long-term low salinity challenges, and narrow-sense heritability estimates were similar for both challenges (short-term $h^2 = 0.35$, long-term $h^2 = 0.4$). Strong phenotypic ($r_S = 0.89$) and genetic ($r_G = 0.81$) correlations for family mortality were found between challenges. Algal clearance metrics over a 24-hour clearance experiment differed among families ($p < 0.001$), but were only weakly associated with family survival in the long-term low salinity exposure (range $p = 0.08 – 0.23$, $R^2 = 5\text{-}10\%$). Growth was negligible during the long-term challenge. However, after being returned to ambient salinities (13), there was no difference in growth rate, wet weight gain, or mortality between oysters from families with low

or high survival in the long-term exposure ($p > 0.05$), indicating that low salinity tolerant oysters can recover following low salinity events. This work shows that a short-term (2 month) low salinity (2.5) challenge at a constant temperature captures the same patterns of family mortality as a 6-month, temperature fluctuating challenge. Additionally, measuring individual oyster clearance rate and the parameters derived from the algal removal curves provide additional insight into the physiological status of oysters under extreme low salinity stress.

Keywords: Oyster aquaculture, heritability, quantitative genetics, physiology, clearance rate, growth

1. Introduction

Environmental extremes associated with climate change pose risks to the United States aquaculture industry. In coastal areas, large freshwater pulses are common following heavy rainfall and storm events (Andrews et al., 1959; Cheng et al., 2015; Du et al., 2021; Engle, 1946; Schubel and Pritchard, 1986; Southworth et al., 2017), or resulting from anthropogenic activities, such as freshwater diversions (Brammer et al., 2007; Butler, 1952, 1949; Gledhill et al., 2020; Gunter, 1953). These pulses of freshwater can expose local eastern oyster (*Crassostrea virginica*) aquaculture operations to extreme low salinity (< 5) conditions for extended periods of time. Eastern oysters perform best at intermediate salinities ranging from 14-28 (Shumway, 1996), and a lower optimal range (~ 9 – 16) has been proposed for populations where freshwater input dominates the hydrodynamics of the system (La Peyre et al., 2016; Lowe et al., 2017; Rybovich et al., 2016). However, severe drops in salinity (< 5) can result in rapid, mass mortality events (Andrews et al., 1959; Beaven, 1946; Du et al., 2021; Gledhill et al., 2020; Munroe et al., 2013; Southworth et al., 2017; Tarnowski, 2020).

Oyster aquaculture operations are negatively impacted by major reductions in growth experienced in low salinity environments (La Peyre et al., 2013; Leonhardt et al., 2017; Loosanoff, 1952; Lowe et al., 2017; Rybovich et al., 2016). Reductions in growth can delay the harvest and selling of a farmer's crop, thereby lowering a farm's profits while also limiting overall farm space due to crop surplus (Hudson, 2019; van Senten et al., 2019). However, environments with salinity < 12 can provide a refuge from some marine predators, such as oyster drills (Manzi, 1970; Melancon, 1990), boring sponges (Hopkins, 1962), mud crabs (MacKenzie, 1970), and black drum (Brown et al., 2008). Additionally, low salinity provides a refuge from the parasites *Haplosporidium nelsoni* and *Perkinsus marinus*, which are the agents of MSX and

92 Dermo disease, respectively, in oysters, and these diseases can cause significant mortality for
93 oyster aquaculture operations (Andrews, 1964; Burreson and Ragone Calvo, 1996; Craig et al.,
94 1989; Shumway, 1996; Tarnowski, 2020).
95 We previously established the feasibility of breeding for survival in extreme low salinity
96 by developing an efficient, lab-based progeny test that resulted in estimated heritability values of
97 ~ 0.4 – 0.5 (mean shell height > 80 mm and mean age ~2-3 years old for oysters challenged in
98 McCarty et al., 2022, 2020). The progeny test was conducted over ~ 1 month at a static salinity
99 (2 – 3) and temperature (27°C) that produced mortality over a relatively short and predictable
100 time frame (substantial mortality began in 6-10 days and reached 23-53% by day 30; McCarty et
101 al., 2020), which is suitable for the typical workflow and demands of a research breeding
102 program. Maintaining a static temperature for low salinity challenges is convenient in a
103 laboratory setting, but the natural environment, where oyster performance is critical for farm
104 productivity, has much more variability in temperature and this variability may alter the outcome
105 of such a challenge. Temperature is the primary factor impacting mortality during low salinity
106 exposure events, with higher temperatures (> 26°C) causing more significant and rapid mortality
107 in lab and field contexts (La Peyre et al., 2013; Loosanoff, 1952; Southworth et al., 2017).
108 Investigating survival during a longer-term and more realistic low salinity exposure with
109 fluctuating temperatures can shed light on the validity of our short-term (30-day) experimental
110 challenge at capturing survival trends that are more representative of field conditions.
111 The first objective of this study was to compare how a longer low salinity challenge with
112 naturally fluctuating (ambient) temperature affects oyster survival and estimates of quantitative
113 genetic parameters for extreme low salinity survival. To do this, we performed two lab-based
114 low salinity challenges (salinity < 3) for 6 and 2 months. During a 6-month challenge at a salinity

of 2.5, temperature was adjusted daily to match that of ambient conditions that year in the field, while temperature remained constant (27 °C) for the 2-month challenge. During the 6-month challenge, clearance metrics and growth were recorded for a subset of individuals to address our second objective: to understand how a key physiological phenotype of oysters (i.e. removal of algae from the water column) may be associated with performance under low salinity stress. Following the 6-month challenge, a recovery experiment was conducted on another subset of individuals from families with high survival to address our third objective: to determine how families that performed best during the 6-month low salinity challenge recovered and performed at ambient salinities more typical of the area (10.42 ± 0.037 SE average daily ambient salinity in Choptank River, Maryland from 2008 – 2021). The recovery experiment is an important examination of potential tradeoffs between low salinity tolerance and performance at more typical, ambient salinities.

2. Methods

2.1 Production of low salinity lines and breeding design

Full-sibling diploid families were created from the low salinity breeding families at the Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science (Allen et al., 2021). These families have been selected for survival at low salinities ~6-15, but no selection has occurred for any traits at an extreme low salinity (< 3). In brief, animals from the ABC low salinity family lines were strip-spawned in mid-April 2018 and mated in a 2 x 2 partial factorial design, where every female was crossed with 2 different males and every male was crossed with 2 different females (Allen et al., 2021). Therefore, some families may share either parent with another family, making them half-siblings. Larvae were reared and fed

following standard ABC protocols (Allen et al., 2021), and individuals from a total of 51 families were transferred from the Coan River, an upstream tributary of the Potomac River in Maryland, USA, to Horn Point Laboratory (HPL), Maryland USA in March 2019. Families were brought immediately into the laboratory and kept in tanks with flow-through, ambient water from the Choptank River, Maryland, USA (salinity 9.8 ± 0.11 standard error (SE), temperature 6.6 ± 0.28 °C SE) until experimentation began in April 2019.

2.2 Laboratory-based low salinity challenges

Two low salinity challenges were conducted following the same procedures described by McCarty et al. (2020), but with a few modifications. Before the challenge began, oysters were approximately 1-year old and averaged 38.10 ± 0.18 mm SE in shell height (family mean shell height, Supplemental Table 1). The first challenge (referred to as the 'long-term' challenge) began on April 1st, 2019 after oysters from the 51 families were acclimated in 3x2 ft Taylor floats (1 inch wire cage ballasted by 4 inch diameter PVC) for a week under laboratory conditions. For each family, oysters were separated into two identically-sized replicates, with total individuals per family ranging from 110–210 total oysters. We included all oysters from ABC to maximize our overall sample size, therefore family and replicate sizes varied. For each family, replicates were randomly assigned to plastic baskets within one of twelve floats, and floats were randomly assigned to one of four tanks to begin the challenge. Salinity was manually decreased gradually over a two-day period to a desired level of 2.5 ± 0.030 SE. Salinity was monitored daily by point sampling using a YSI-85 handheld multimeter (YSI Incorporated, Yellow Springs, OH, USA) and adjusted by mixing ambient, Choptank River water (salinity 6.8 ± 0.11 SE) and well water (salinity 0) to maintain levels within 0.5 of the target salinity under

flow-through conditions. Temperature was checked and adjusted daily by heating or cooling the well water to track that of local ambient conditions in the Choptank River. During the long-term challenge, temperature gradually rose and fluctuated from 7.9°C to 30.3°C, with temperature peaking on July 21st. Oysters remained at the desired salinity (2.5) and corresponding ambient temperature for 168 days (~ 6 months). Across all tanks, water flow averaged 0.476 L s^{-1} ± 0.00928 SE and dissolved oxygen averaged 6.58 mg L^{-1} ± 0.0537 SE throughout the challenge. Oysters were inspected weekly to check for mortality and shell height was recorded for all dead individuals. Shell height was measured using Vernier dial calipers as the length in mm from oyster hinge to bill. During the challenge, flow-through Choptank River water supplied some natural phytoplankton, but at least half of the water entering the experimental tanks was well water (i.e. no phytoplankton). Therefore, diet was supplemented every 3 days with Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA) at a ration of 1.5% of oyster dry weight according to Reed Mariculture's suggested feeding rates (shell height was converted to dry biomass using the power equation; Southworth et al., 2010), as was done in previous studies and challenges (McCarty 2020, 2022). Flowing river and well water were shut off for a period of two hours during supplemental feeding. Tanks were drained and scrubbed weekly to reduce accumulation of sediment and floats were rotated among tanks once a week after mortality was assessed. Once a month for a total of 6 sampling time points, a subset of 25 individuals from each replicate (total of 50 per family) were measured for shell height to track growth throughout the duration of the experiment.

A separate control tank containing 500 oysters, 70 individuals each from six of the half-sibling families and 80 wild oysters from the Choptank River (Shoal's Creek, MD), was set up with continuous, flow-through river water at ambient salinity and temperature ranging from 5 –

184 11.5 and 7.9°C – 30.3°C, respectively, for the duration of the long-term exposure. Dissolved
185 oxygen averaged 7.18 mg L^{-1} ± 0.129 SE in the control tank during the 6-month exposure period.
186 Daily mortality was not measured for these individuals considering the very minimal mortality
187 observed in two previous experimental challenges (McCarty et al., 2020), but monthly
188 assessment of individual growth revealed very minimal, if any, mortality. Growth was assessed
189 monthly by random selection of 25 oysters from each of the half-sibling families for a total of
190 150 individuals.
191 On April 29, 2019, 30 oysters from each replicate, totaling 60 per family, were removed
192 from the long-term low salinity challenge and transferred for a separate, 'short-term' low salinity
193 challenge. Individuals were removed from the long-term challenge at a salinity of 2.7 ± 0.10 SE
194 and temperature of 17.9°C ± 0.0250 SE on transfer day (day 26 of long-term exposure). Values
195 represent the average salinity and temperature across the four experimental tanks oysters were
196 removed from. Average mortality (across all families) before being transferred to the short-term
197 challenge was minimal, at 1.82%, likely due to low ambient temperature (<20°C; e.g. McFarland
198 et al., 2022; Southworth et al., 2017). For the short-term challenge, nine plastic baskets in three
199 Taylor floats were divided into two halves using 1-inch vinyl coated wire mesh cage material.
200 Families (no replicates) were randomly assigned to a plastic basket section and all three floats
201 were placed in the same 6-ft diameter tank already at the target salinity (~ 2.5) and temperature
202 (~ 27°C). Salinity and temperature were maintained at 2.41 ± 0.396 SE and 27.8°C ± 0.188 SE,
203 respectively, throughout the short-term challenge. Salinity and temperature levels were assessed
204 daily and adjusted to maintain within 1 ppt and 1°C of target levels. Dissolved oxygen was
205 monitored daily and averaged 6.41 mg L^{-1} ± 0.0631 SE throughout the challenge. Floats were
206 pulled and mortality was checked every 4 days for 60 days. Feeding was identical to the long-

term challenge, except supplementation with Shellfish Diet 1800® occurred every other day to replicate the feeding schedule during the two previous challenges in McCarty et al. (2020). Water flowed through the tank at an average of 0.527 L s^{-1} ± 0.0108 SE throughout the short-term challenge.

2.3 Statistical analyses for low salinity challenges

An ANCOVA was conducted to analyze the effect of family (51 families) and sampling month (7 time points) on growth, measured as individual height (mm; 50 individuals per family) during the long-term low salinity challenge in the 'stats' package (version 4.0.2) in the R statistical software (version 3.6.1; Core Development Team, 2020). A separate ANCOVA was conducted for individuals (N = 25 per family) from the six families in the control tank to assess differences in growth among families held at ambient conditions. Correlations between family mean shell height (mm) before the exposure began (March) and family cumulative survival in the long-term challenge were assessed using a Spearman's rank correlation test in the 'Hmisc' package (version 4.3; Harrell, 2021). All computations were performed using the R statistical software (R version 3.6.1; Core Development Team, 2020).

2.4 Estimation of quantitative genetic parameters

Underlying narrow-sense heritability (h^2) was estimated for the liability (survival) in each challenge independently using ASReml-R (Butler et al., 2017; McCarty et al., 2020; Wilson et al., 2010):

$$l_i = \mu + a_i + e_i$$

where, for oyster i, l_i is the survival phenotype (0 or 1), μ is the average population survival, a_i is the additive genetic effect of the alleles on the phenotype, and e_i is the residual effect accounting for the remaining variation. Equations to estimate genetic parameters and how to convert from the observed to liability scale are described in detail in our previous study (McCarty et al., 2020). Replicate and float were incorporated into each model accordingly as fixed effects to account for any additional variation introduced from the experimental blocking structure. Phenotypic correlations (r_s) between the two challenges were investigated using a Spearman's rank correlation test using the 'Hmisc' package (version 4.3; Harrell, 2021), and a bivariate model was run to investigate genetic correlations (r_g) between the two challenges using ASReml-R (Wilson et al., 2010). All statistical analyses were conducted using the R Statistical Software (R version 3.6.1; Core Development Team, 2020).

2.5 Clearance experiment

Removal of algae from the water column was measured for individual oysters at four time points during the long-term exposure. Oysters were exposed to the long-term challenge for five weeks prior to these measurements. Three to five individuals per family for 31 of the 51 families were examined for clearance capabilities across four days: May 13, 20, 22, and June 20. Individuals were sampled over multiple days to maximize the number of families and individuals sampled. Families were chosen based on survival in the long-term challenge, ensuring families with both high and low survival rates were selected. Seven of the 31 families sampled had high survival at the end of the long-term exposure, meaning their family cumulative survival was in the top 10 of all families (88 – 97% cumulative survival). Eight of the families sampled had low survival (bottom 10 of families) in the long-term challenge (35 – 63% cumulative survival) and

252 the remaining 16 families had cumulative survival values between the top 10 and bottom 10
253 performing families. Artificial seawater for the experimental beakers was produced by salting
254 deionized water to a salinity of 2.5 with Crystal Sea® Marinemix (Marine Enterprises,
255 Baltimore, MD), and a bubbler was added to each beaker to supply air (oxygen) and keep
256 phytoplankton mixed. Water temperatures ranged from 20.7°C to 24.7°C across the four
257 experimental days. A subset of individuals (N=5) was tested on multiple sampling days to
258 investigate how day and temperature affected individual clearance performance.
259 On each experimental day, live algae (*Chaetoceros muelleri*) from the Horn Point Oyster
260 Hatchery (Cambridge, MD) were added to beakers. Algae were added to each beaker containing
261 800 mL of artificial seawater in 1 mL increments until the FluoroSense™ Handheld Fluorometer
262 (model # 2860-000-C, Turner Designs, San Jose, CA) chlorophyll a concentration reading
263 reached just below the maximum detection limit (199 μg L^{-1}). Subsamples of water were
264 collected from the beaker after each incremental algae addition and algal cells were counted
265 (cells mL^{-1}) in triplicate on a Levy Improved Neubauer hemocytometer (Hausser Scientific, PA)
266 using an Olympus BX41 Phase Contrast & Darkfield Microscope (Olympus©) at 20X
267 magnification. To determine the statistical relationship between chlorophyll a concentration (μg
268 L^{-1}) measured by the fluorometer and the mean cell concentration (cells mL^{-1}) measured from
269 triplicate hemocytometer counts, standard curves were calculated using ordinary least squares
270 regressions through the origin in the 'stats' package (version 4.0.2) in the R statistical software
271 (version 3.6.1; Core Development Team, 2020). Separate serial dilutions and standard curves
272 were calculated for each feeding day, except for the last day (June 20th, Figure 1). The
273 relationship between chlorophyll (μg L^{-1}) and algal concentration (cells mL^{-1}) was strong (R^2 >

0.94) and did not differ among days (ANCOVA $p > 0.15$), thus data were combined for the standard curve used on the 4th (June 20th) experimental feeding day (Figure 1).

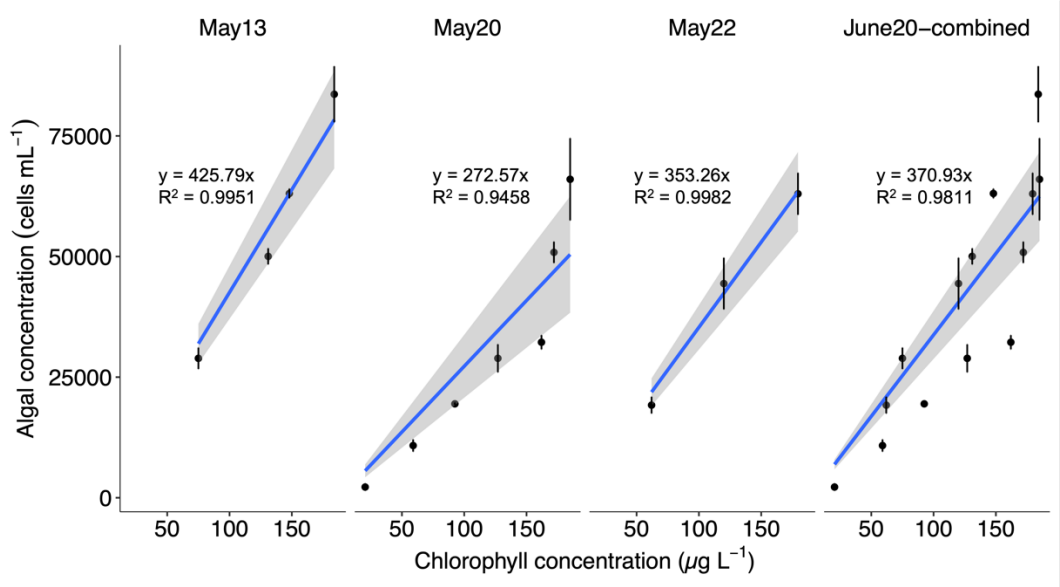

Figure 1. Relationship between algal (average of triplicate hand counts; cells mL^{-1}) and chlorophyll a (μg L^{-1}) concentrations for the serial dilutions from the clearance experiments. The equations determined from the ordinary least squares regression lines (in plot) were forced through the origin and the shaded region represents the 95% confidence interval of the regression equation. The plot and regression equation for June 20th include all data from the three previous experiments (i.e. all data combined). Error bars represent SE for the triplicate hand counts.

After the correct volume of algae was added to each beaker (~ 9 – 11 mL of algae; ~75,000 cells mL^{-1}), each individual oyster was removed from the long-term low salinity challenge and gently scrubbed to remove living organisms and detritus. Oysters were then placed into individual beakers and beakers were randomly positioned on the benchtop. Fluorometer readings were taken in duplicate for each beaker by lowering the FluoroSense™ to the designated line on the device (~ 2 inches below the surface) at time 0 when oysters were first

placed in the beaker, and at 3, 6, 9, 12, 15, and 24 hours after experimentation began. Before returning individuals to the long-term challenge after sampling, Floy® Custom UV Protected Vinyl Laminated oval shellfish tags (Seattle, WA) were adhered near the hinge of each organism using Loctite® Super Glue Liquid (Westlake, OH) to track individual survival after being returned to experimental tanks. Triplicate beakers with no oysters were used as a control for each sampling timepoint on each experimentation day to account for any cell sinking or sticking to the sides of the beaker (ΔA in clearance rate equation below).

2.6 Statistical analysis of clearance experiment

Fluorometer readings (chlorophyll a; μg L^{-1}) were converted to cellular abundance estimates (cells mL^{-1}) using the standard curves described above. For each individual oyster, three clearance metrics were calculated for subsequent analysis: average clearance rate (CR_{avg}), maximum algal removal rate (R_{max}), and time to 50% algal depletion (D_{50}). These metrics were decided upon in hopes of capturing the observed sigmoidal trends in algal removal over the experimental period (Results, Figure 5). One-way ANOVAs were conducted to assess differences in the three feeding metrics among families. We then took an average of these three metrics for all individuals within a family to investigate statistical associations between family clearance metrics and family survival in the long-term low salinity challenge.

To determine the average clearance rate for each individual, clearance rates (CR, L hr^{-1}) were calculated at each time interval (i.e. 0–3 hours, 3–6 hours, etc.) (Coughlan, 1969; McFarland et al., 2013; Riisgård, 1988):

$$CR = \left(\frac{V}{t} * \left(\ln\left(\frac{C_0}{C_t}\right) - \Delta A \right) \right)$$

where V= volume of water in liters, t is elapsed time in hours, C_0 is the initial concentration (cells mL^{-1}), C_t the algal concentration at the given sampling time, and ΔA is the average algal cell loss across the three control jars for the specified time interval (i.e. $\ln(\frac{A_0}{A_t})$). An average clearance rate was calculated for each oyster from the series of clearance rates (i.e. 0–3 hours, 3–6 hours, etc.). Clearance rates for time intervals where algae was already depleted, and therefore fluorescence was not measured, were excluded from the overall average. Each individual average was normalized for size using the average experimental oyster shell height (Bayne, 2017; Cranford et al., 2016, 2011):

$$CR_{avg\ height} = (H_{std}/H_{ind})^{1.78} * CR$$

where H_{std} is 38 mm (average shell height of the experimental oysters), H_{ind} is the shell height (mm) of each individual oyster, and CR is the average clearance rate (L hr^{-1}) from the prior equation. Normalization was conducted using shell height because there were not enough oysters for destructive, dry weight sampling, and shell height is known to correlate well with dry weight (ex. Cornwell et al., 2016; Mann and Evans, 1998; Paynter and Dimichele, 1990).

Locally estimated scatterplot smoothing (LOESS) splines were used to determine two additional clearance metrics for each individual: the time to deplete half (50%) of the algae in each beaker (D_{50}) and the maximum algal removal rate (R_{max}). For each individual, LOESS splines were estimated for the concentration of algae (cells mL^{-1}) present over the 24-hour period using the 'stats' package (version 4.0.2) in the R statistical software (version 3.6.1; Core Development Team, 2020). All LOESS curves were estimated with a span of 1 for maximum smoothing of each curve. The splines were used to determine the time at which 50% of the starting algae concentration was depleted for each individual oyster. Maximum algal removal rate for each individual oyster was estimated as the derivative at the steepest part of the LOESS

335 curve. The absolute value of R_{max} was used to make this value positive (i.e. the slope or rate of
336 algal depletion is a negative value) and individual D_{50} and R_{max} were divided by individual shell
337 height for size normalization.
338 A family average was taken for each of the three clearance metrics. Ordinary least square
339 regressions using 'glm' ('stats' package version 4.0.2, R version 3.6.1; Core Development Team,
340 2020) were performed to determine if family CR_{avg} (normalized for height, referred to as CR_{avg}
341 from here on), family D_{50}, and family R_{max} were associated with family cumulative survival in
342 the long-term (6-month) low salinity challenge. Family cumulative survival (proportion between
343 0 and 1) was logit transformed before regression analyses to make the variable normally
344 distributed. Correlations were conducted between the three clearance metrics for each family
345 ('stats' package version 4.0.2, R version 3.6.1; Core Development Team, 2020) to assess the
346 relationship between the clearance metrics. Paired t-tests were run on the three clearance metrics
347 (CR_{avg}, D_{50}, and R_{max}) for individuals (N = 5) that were repeated on multiple days to assess the
348 effect of experimental day on the measured metric (i.e. effect of varying temperature and other
349 experimental design factors). Lastly, Welch's two sample t-tests were conducted to assess
350 differences in the three clearance metrics for individuals that survived (N=143) compared to
351 those that died (N=35) after the long-term challenge ('stats' package version 4.0.2, R version
352 3.6.1; Core Development Team, 2020).
353
354 2.7 Individual oyster recovery after long-term low salinity exposure
355 At the culmination of the long-term low salinity challenge, 35-128 oysters from 11
356 families and from one of the control families (N = 1,070 oysters total) were retained to assess
357 how oysters performed when re-introduced to more favorable, ambient salinity conditions.

Oysters from some families were retained for use in other projects, hence the uneven stocking density. The 11 families chosen for this experiment demonstrated just-below average to high survival in the short or long-term challenge: long-term mortality among the 11 families ranged from 20% (Fam 23; 29th (of 51) most mortality) to 2.7% (Fam. 39; least mortality of 51 families; Figure 3 aqua dots). These families were selected because we were explicitly interested in how families that had very high survival at extreme low salinity (i.e. the families we would select for breeding) would perform at ambient salinities after a low salinity exposure (i.e. is there a tradeoff in growth/survival of high performance families after a low salinity exposure). All families were divided into two replicates except for families 6, 23, and 39 where replication was not possible due to low sample sizes, and replicate groups were returned to the aforementioned plastic baskets secured to three modified Taylor floats. Floats were returned to a 6 ft diameter (~1800 liter) tank in the laboratory with ambient flow-through Choptank River water for a period of one month. Salinity and water temperature during the recovery assessment period were 11.2 ± 0.0425 SE and 20.5°C ± 0.427 SE, respectively. Supplemental feeding ceased during this period of study and naturally occurring algae and organic matter provided the sole source of food for the oysters. Twice weekly, the tank was fully drained and cleaned to remove biodeposits and other organic matter.

For all experimental individuals, oyster shell height (mm, hinge to bill) was measured before (October 1st), after two weeks (October 18th), and after one month (October 31st) of re-introduction to the ambient conditions using Vernier dial calipers. Oyster mortality was also assessed bi-weekly, and dead oysters were removed from the baskets. Ten oysters per replicate (N = 20 for all families except for families 6, 23, and 39 where N = 10) were patted dry and labeled with a SHARPIE® Industrial Pro permanent marker. Oyster whole wet weight was

assessed for these labeled oysters before introduction to ambient conditions and again after the end of the recovery period by weighing, to the nearest milligram, on an Ohaus Discovery analytical electronic balance (Model DV114C, Ohaus Corporation, Parsippany, NJ, USA) after oysters were patted dry and allowed to sit in ambient laboratory conditions for 24 hours. Two separate ANCOVAs were conducted to compare the slopes (growth rate and wet weight gain) among families (11 from the long-term challenge and 1 from the ambient control) and sampling day during the recovery experiment. To compare the mortality (proportion of dead) among families during the recovery experiment, the Marascuilo procedure was conducted (Marascuilo and McSweeney, 1967). To determine if growth rate, wet weight gain, and mortality during the recovery experiment were associated with long-term low salinity challenge survival, family growth rate, rate of wet weight gain (estimates from the ANCOVAs), and mean family survival during the recovery experiment were regressed on family survival in the long-term low salinity challenge using 'glm' in the R statistical software ('stats' package version 4.0.2, R version 3.6.1; Development Core Team, 2020).

3. Results

3.1 Experimental results and trends in mortality for the two low salinity challenges

Differential mortality was observed among the 51 half-sibling families during both the short-term challenge at a salinity of ~2.5 and temperature of 27°C and during the long-term challenge at a salinity of ~2.5 and fluctuating temperature. During the 6-month challenge, a total of 1,712 oysters died representing 25.8% of the total experimental population. Two spikes (peaks) in mortality were observed during the long-term exposure, one on day 61 (June 3, 134 dead oysters, 8% of mortality) at a temperature of 24.1 °C, and the other spike occurred on day

112 (July 24, 235 dead oysters, 14% of mortality) at a temperature of 28 °C (Figure 2B). Temperature was greater than 27°C for 27 days, beginning on day 86, before the second mortality spike was observed on day 112. During the short-term challenge, no mortality was experienced during the first 10 days of exposure. Mortality peaked on day 43 of exposure with 250 oysters recorded dead across all families (39% of total mortality, Figure 2A). A total of 635 oysters were recorded dead at the end of the 2-month challenge, representing 21% of the total experimental population.

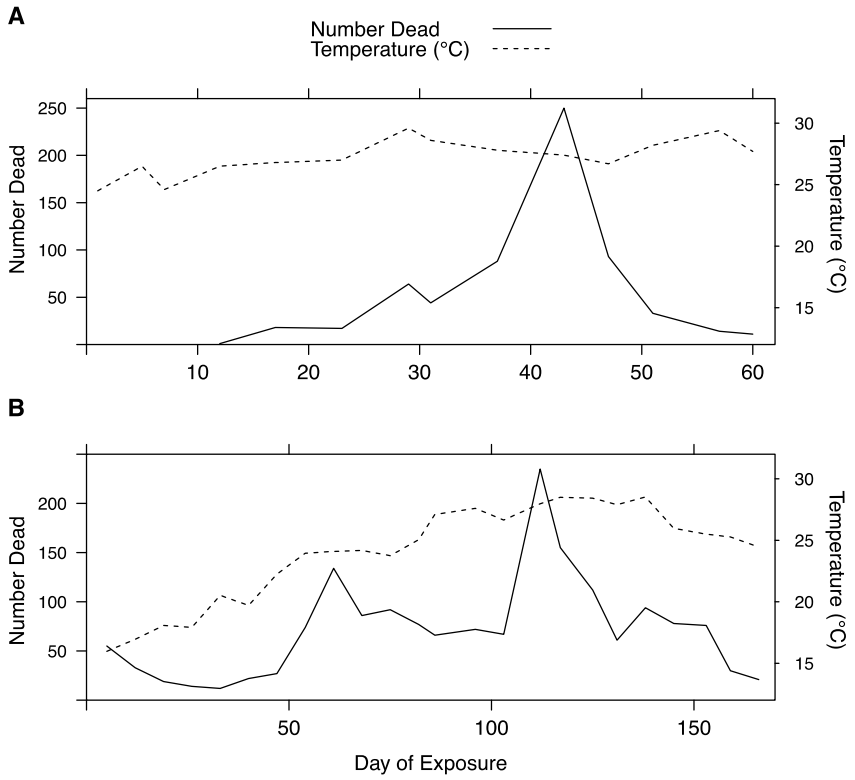

Figure 2. Number of dead individuals during the two low salinity experimental challenges. Number dead (solid black line) and temperature (°C, dotted black line) during the A) short-term, 60-day challenge and B) long-term, 168-day challenge. Mortality was assessed every 4 days for the short-term challenge and daily for the long-term challenge. Temperature was adjusted to track ambient levels during the long-term challenge.

There was no statistically significant interaction between the effect of family and sampling month on individual height (ANCOVA, $F_{300,17328} = 1.062$, $p = 0.221$), and results suggest that oysters lost shell height during the low salinity exposure. Main effect analysis showed a statistically significant effect of both family (ANCOVA, $F_{50, 17328} = 98.12$, $p < 0.001$) and sampling month (ANCOVA, $F_{6, 17328} = 56.80$, $p < 0.001$) on individual height, mainly due to the significant differences in family height before the long-term challenge began (ANOVA, $F_{50,2493} = 18.12$, $p < 0.001$). There was a statistically significant interaction between family and sampling month on individual height (ANCOVA, $F_{30,1008} = 2.4465$, $p < 0.001$) in the control tank at ambient salinity and families in the control tank grew an average of 26.1 ± 1.34 SE mm over the 6 months. There was no correlation between mean family height pre-exposure (March, mm) and family cumulative survival in the long-term challenge ($r_s = -0.168$, $p = 0.236$).

3.2 Narrow-sense heritability (h^2) and correlations across challenges

In the long-term challenge, family mortality ranged from 2.72% to 65.3% with a mean family cumulative mortality of 25.2% (aqua dots, Figure 3). In the short-term challenge, mortality among families ranged from 0% to 63.3% with a mean family cumulative mortality of 20.75% (black dots, Figure 3). Family survival was similar between the two challenges (Figure 3). Nine of the ten families with the highest mortality (lowest surviving ten families) in the long-term challenge were also in the top ten for highest mortality in the short-term challenge. Similarly, six of the ten families with the lowest mortality (highest surviving ten families) in the long-term challenge were also in the top ten for lowest mortality in the short-term challenge.

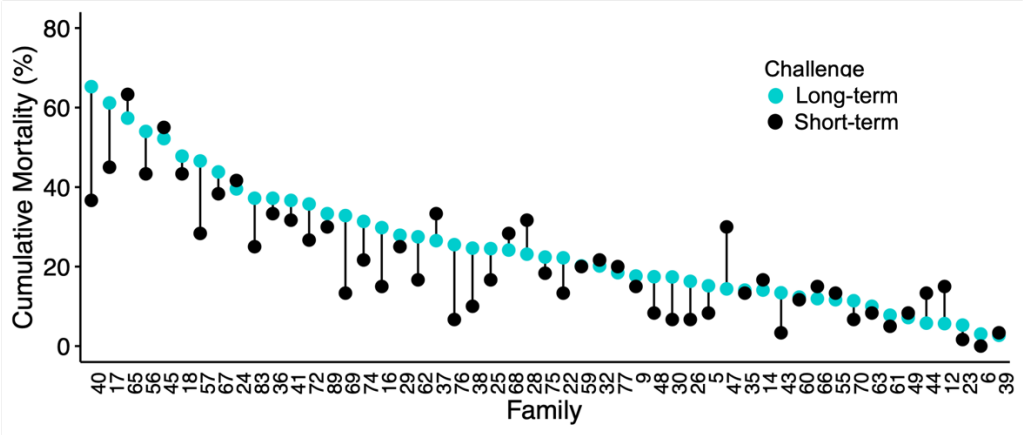

Figure 3. Lollipop plot depicting similar family mortality across the two low salinity challenges. Cumulative family mortality (%) from the long-term (aqua dots) and short-term challenge (black dots).

Underlying narrow-sense heritability estimates were moderate for both challenges and were both significantly different than zero. Narrow-sense heritability was 0.3505 ± 0.026 SE for the short-term challenge, and 0.4093 ± 0.036 SE for the long-term challenge (Table 1). There was a large and statistically significant phenotypic correlation for family mortality (% mortality) between the two challenges ($r_S = 0.81$; $p < 0.0001$; Figure 4, Table 1). Similarly, the genetic correlation between family mortality for the two experiments was also very large and statistically significant (0.89 ± 0.07 SE, Table 1).

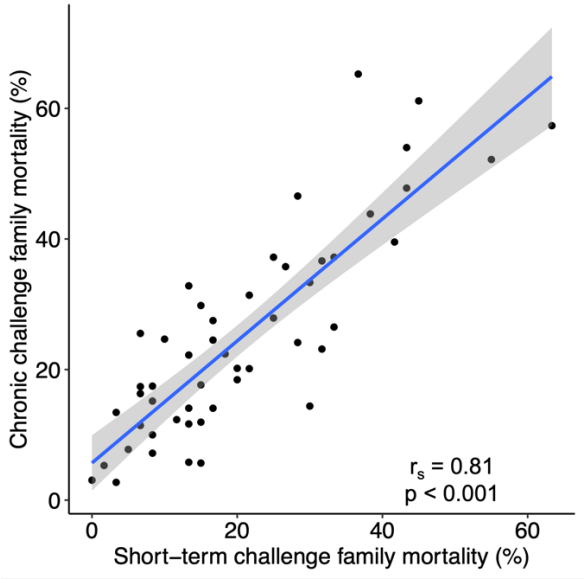

Figure 4. Scatter plot displaying correlation in cumulative mean family mortality (%) between the long-term and short-term challenges. Spearman rank correlation coefficient (r_S) and significance value is displayed in the bottom right, and shading represents the 95% confidence interval for the ordinary least square regression equation.

Table 1. Narrow-sense heritability (h^2 ± SE) and correlations between mortality in the two challenges. Phenotypic correlations (r_S), and genotypic correlations (r_G ± SE) using the animal model in ASReml-R.

	h^2	r_G	r_S
Long-term	0.4093 ± 0.036	0.89 ± 0.07	0.814
Short-term	0.3505 ± 0.026		

3.3 Clearance experiment analysis

Most oysters among the 31 families examined removed algae at a salinity of 2.5, reducing the concentration of algae in the experimental beaker over the 24-hour sampling period. Clearance metrics for oysters measured (repeatedly) across multiple experimental days (N = 5) did not vary significantly (paired t-tests, $p > 0.05$; maximum algal removal rate: R_{max}, $t(4) = 0.523$; average clearance rate: CR_{avg}, $t(4) = -2.57$; time to half: D_{50}, $t(4) = 2.14$). The rate of

decline in phytoplankton concentration (cells mL^{-1}) over time generally demonstrated a sigmoidal relationship, in which algal concentration was high and unchanged initially (oysters slow to clear phytoplankton during the first few time points) before dropping over time as oysters cleared algae from the water column (Figure 5). However, there were interesting patterns in algal removal that varied between oyster, family group, and family survival in the chronic challenge (Figure 5). Some oysters removed algae at faster rates than others and some oysters failed to reach 50% algae depletion (black curves, Figure 5).

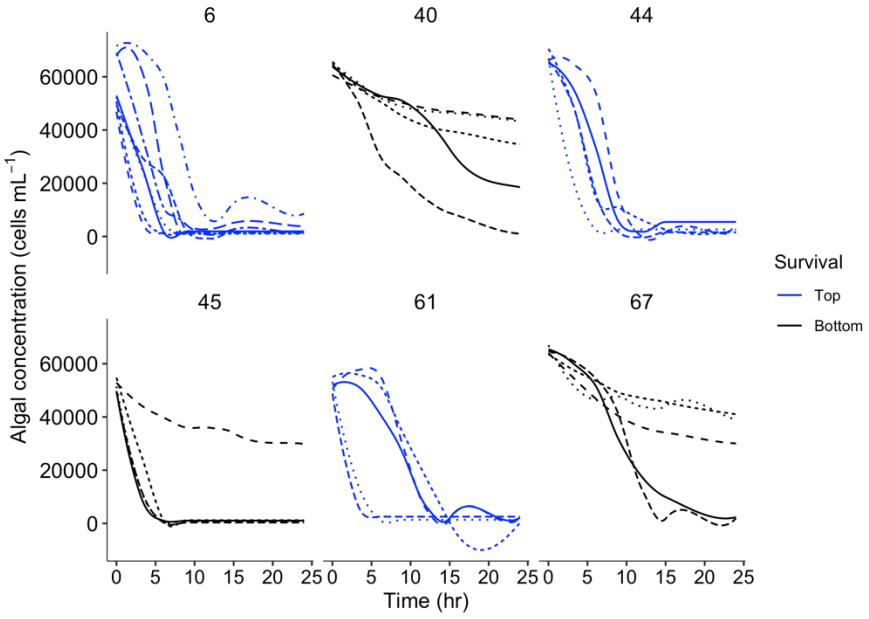

Figure 5. Algae concentration (cells mL^{-1}) over 24 hours for 6 families at a salinity of 2.5. Individual replicates are depicted by different line types and grouped together by family. Lines are color-coded based on the relative ranking of survival across all families, black indicating lowest survival (bottom ten surviving families: families 40, 45, 67) and blue indicating highest ranking survival (top ten surviving families: families 6, 44, 61).

One-way ANOVAs revealed statistically significant differences in individual clearance metrics between families (CR_{avg}: $F_{(30,151)} = 3.027$, $p < 0.001$; R_{max}: $F_{(30,151)} = 2.686$, $p < 0.001$; D_{50}: $F_{(29,119)} = 2.547$, $p < 0.001$). There was no significant association between family average clearance rate normalized for height (CR_{avg}; L hr^{-1}) and family mean survival (logit transformed) in the long-term (6-month) low salinity challenge ($p = 0.23$; Figure 6A). Family CR_{avg} ranged from -39.57 – 517.6 L hr^{-1}, with an average family CR_{avg} of 178.7 L hr^{-1}. Family maximum algae removal rate normalized for individual height ($R_{max,}$ cells hr^{-1} mm^{-1}) was close to, but not significantly associated with survival in the long-term challenge at the alpha 0.05 level ($p = 0.08$) and only explained a small proportion of the variation (10%, Figure 6B). Family R_{max} ranged from 76.3 – 290 cells hr^{-1} mm^{-1} with an average family R_{max} of 173 cells hr^{-1} mm^{-1}. Family average time to deplete 50% of the starting algae concentration normalized for individual height (D_{50}; h mm^{-1}) was close to, but not significantly associated with survival ($p = 0.09$) and explained a small portion of variation (10%; Figure 6C). Family D_{50} ranged from 0.063 to 0.495 hr mm^{-1} with an average family D_{50} 0.260 hr mm^{-1}.

A total of 35 (19.7%) individuals labeled for the clearance experiment died after being returned to the long-term challenge. Of the dead oysters, 57% (20/35) came from families with the highest mortality (lowest surviving ten families) and 0.06% (2/30) came from families with the highest survival (top surviving ten families) in the chronic challenge. There was a significant difference in all three clearance metrics between oysters that died versus those that survived the long-term challenge ($CR_{avg:}$ $t(50.21) = -2.403$, $p = 0.02$; D_{50}: $t(27.23) = 2.079$, $p = 0.047$; R_{max}: $t(50.97) = - 4.079$, $p < 0.001$). Oysters that survived had higher mean CR_{avg} = 235.02 L hr^{-1}, lower D_{50} = 0.225 h mm^{-1}, and higher R_{max} = 194 cells hr^{-1} mm^{-1}, while oysters that died had lower mean CR_{avg} = 116.5 L hr^{-1}, higher D_{50} = 0.314 h mm^{-1}, and lower R_{max} = 128 cells hr^{-1} mm^{-}

[1]. Thirteen oysters that died (37%) never reached the 50% depletion mark. Clearance metrics were moderately to highly correlated to one another (r = 0.55 – 0.78, all p-values < 0.01; Figure 6D - F).

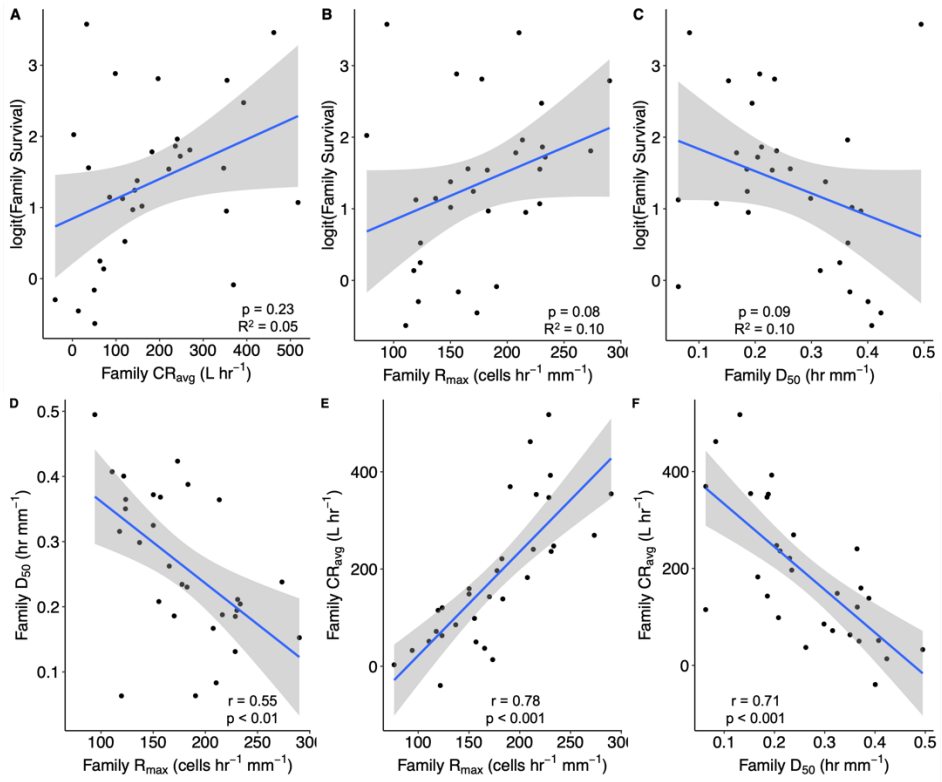

Figure 6. Scatter plots and ordinary least square regression lines of family mean survival (logit transformed) against A) family average clearance rate ($CR_{avg,}$ L hr^{-1}), B) family average maximum algal removal rate (R_{max}, cells hr^{-1} mm^{-1}), and C) average time for each family to deplete 50% of the starting algae concentration (D_{50}, hr mm^{-1}). Regressions between the three clearance metrics are displayed in the bottom row with the associated correlation coefficients (D-F). D) Family D_{50} regressed against family R_{max} (cells hr^{-1} mm^{-1}), and family CR_{avg} (L hr^{-1}) against E) R_{max} (cells hr^{-1} mm^{-1}) and F) family D_{50} (hr mm^{-1}). Grey areas indicate the 95% confidence interval of the regression equations.

3.4 Oyster recovery after extreme low salinity

Growth was negligible during the long-term challenge for the 11 families included in the recovery experiment (Figure 7A, March – Sept), but all 11 families resumed growth within two weeks after being returned to ambient conditions on October 1 (Figure 7A shaded, 7B). During the 1-month recovery period, growth for the 11 families mirrored that of the oysters from the ambient control tank (family 60C, Figure 7A shaded & 7B) and ranged from 0.21 – 0.34 mm day^{-1} with a mean family growth of 0.25 mm day^{-1}. Oysters from the 11 families had an average shell height of 33.70 mm ± 0.226 SE and wet weight of 4.14 ± 0.165 SE grams at the start of the recovery period and gained an average of 2.85 ± 0.097 SE grams wet weight over the 1-month period. Oysters from the family held in ambient conditions during the long-term challenge (control tank, family 60C) were larger at the beginning of the recovery experiment – average shell height of 64.0 ± 1.19 SE mm and wet weight of 19.2 ± 1.65 SE grams – and gained more wet weight, average of 7.21 ± 0.360 SE grams, over the 1-month period. During the recovery experiment, there was no statistically significant interaction between sampling day and family on individual height (ANCOVA, $F_{11, 3156} = 0.4769$, $p = 0.919$) nor weight (ANCOVA, $F_{11, 368} = 1.536$, $p = 0.116$), indicating that there was no significant difference in shell growth rate or wet weight gain (slope) among families tested. For both shell height and wet weight, main effect analysis showed a statistically significant effect of both family (ANCOVA, $F_{11, 3156} = 232.8$, $p < 0.001$ and $F_{11, 368} = 73.74$, $p < 0.001$, respectively) and sampling day (ANCOVA, $F_{1, 3156} = 602.2$, $p < 0.001$ and $F_{1, 368} = 120.4$, $p < 0.001$, respectively), however, this was due to the significant differences in family height and weight at the beginning of the experiment (ANOVA, $F_{11,1059} = 99.66$, $p < 0.001$ and $F_{11,188} = 39.66$, $p < 0.001$, respectively). There was no significant association between family survival in the long-term exposure and family growth rate ($p = 0.688$,

$R^2 = 0.0211$) or wet weight gain ($p = 0.989$, $R^2 = 0$) during the recovery experiment. Recovery mortality ranged from 0 - 31.4% for the 12 families (11 families from long-term challenge and 1 family from the ambient control). Pairwise comparisons between the proportion dead for each family indicated no significant differences among families (Marascuilo procedure, $p > 0.05$ for all comparisons). Additionally, there was no significant association between family survival in the long-term exposure and family mortality during recovery ($p = 0.465$, $R^2 = 0.06$).

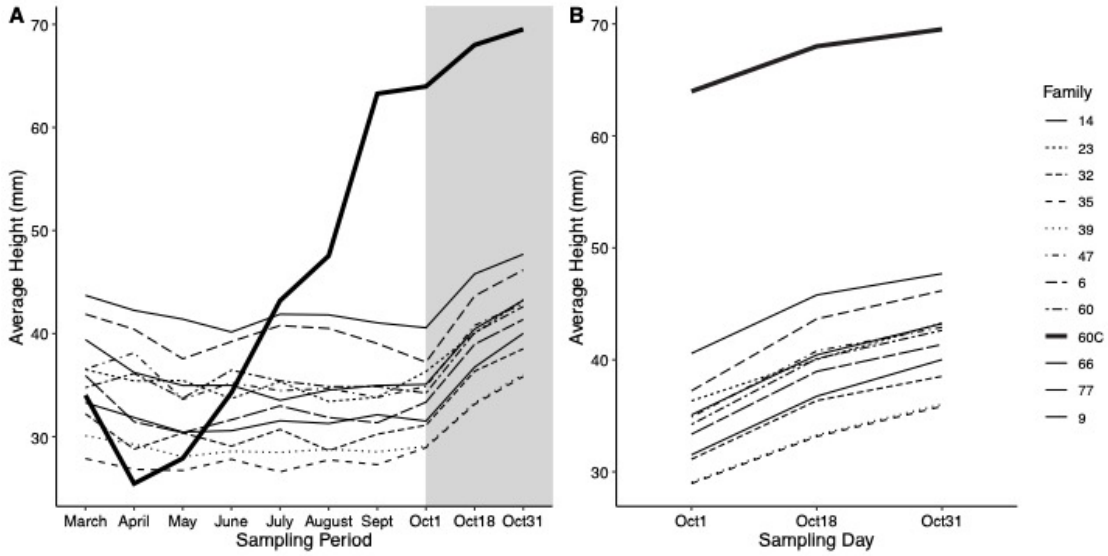

Figure 7. Average shell height (mm) for each family during the long-term low salinity challenge and the recovery experiment. Panel A shows shell height before (March) and during the long-term low salinity challenge (April – Sept), as well as during the recovery experiment (shaded region, Oct 1 - Oct 31). Panel B shows the average shell height for families explicitly during the recovery experiment (shaded region on panel A, Oct 1 - Oct 31). Each family is represented by a different line type, and family 60C (thick black line) was exposed to ambient conditions in the control tank.

4. Discussion

A short-term (2 month) and long-term (6 month) challenge at extreme low salinity (< 3) were conducted to investigate the effect of challenge duration and temperature on oyster extreme low salinity survival. Similar to results from previous extreme low salinity challenges (McCarty et al., 2022, 2020), mortality varied among families in the two challenges and narrow-sense heritability estimates were moderate and significantly different from zero. Family mortality was highly correlated between the short-term and long-term challenges, suggesting that an extreme low salinity challenge at a single high temperature (27°C) captures similar mortality trends as the more realistic, 6-month challenge where temperature was adjusted daily to mimic the natural, seasonal variation in temperature experienced from spring to fall. Results from the clearance experiment revealed differences in algal clearance, quantified as three metrics (average clearance rate: CR_{avg}, maximum algal removal rate: R_{max}, and time to half: D_{50}), among individuals and families when exposed to an extreme low salinity (< 3). Below, we discuss the biological and practical implications of the long-term versus short-term challenge results. We also suggest potential next steps from the clearance experiments and highlight the ability of oysters to recover from long-term low salinity exposure regardless of their family survival in the long-term challenge.

4.1 Short vs. long-term low salinity challenges and comparisons to previous challenges

A primary goal of this study was to assess how a longer-term low salinity challenge with a more natural (ambient) temperature regime, which is more realistic of typical field conditions, would impact overall results and family-specific mortality. Results were similar across the short-term and long-term challenges. Family mortality was highly correlated across the two challenges (> 0.8 for both the phenotypic and genetic correlations) and the rank of family survival was very

similar between the challenges (Figure 3 & 4). Additionally, heritability estimates were similar and moderate for both experiments (long-term $h^2 = 0.4$, short-term $h^2 = 0.35$). The cumulative mortality observed during our two challenges (21 and 25.8%) and the two heritability estimates ($h^2 = 0.35$ and 0.4) are very similar to values previously observed during a low salinity experimental challenge with half-sibling families (spring 2018 challenge cumulative mortality 23% and $h^2 \cong 0.4$, McCarty et al., 2020). Based on these results, a progeny test for low salinity survival in young oysters (< 40 mm) can be conducted using a 2-month experimental exposure at fixed salinity (2.5) and temperature (27°C), which is operationally easier to implement than a 6-month challenge varying water temperature to match changing ambient conditions.

The reduced cumulative mortality observed during the long-term challenge compared to two previous low salinity summer challenges conducted in our lab (McCarty et al., 2022, 2020) may be a result of the ambient temperature and reproductive state of the oysters when the challenge began. In the long-term challenge, the overall cumulative mortality (25.8%) was not nearly as large as previously reported during mid-summer challenges (53% cumulative mortality across families in July-August 2018, McCarty et al., 2020, and nearly 100% cumulative mortality in F_2 families in June – July 2018, McCarty et al., 2022). However, our 2018 challenge (reported in McCarty et al., 2022) used inbred (F_2) oysters, which likely explains the much greater mortality observed. In addition, the long-term challenge began on April 1st when ambient temperature was low (7.9°C), while previous summer challenges began when the temperature was already elevated (~24°C on May 28 and ~26°C on July 17 in 2018, McCarty et al., 2020, 2022). In previous challenges, oyster gonads were likely mature or maturing and spawning may have already been occurring due to the high ambient temperatures when oysters entered the challenges, which could cause increased physiological stress and higher mortality rates (Lambert

et al., 2008; Li et al., 2007; Samain and McCombie, 2008). In the long-term challenge (this experiment), oysters should have been undergoing advanced gametogenesis at 1-year old and ~ 40 mm by July (Galtsoff, 1964), but exposure to a salinity of 2.5 in April, when temperatures were low, may have slowed or arrested gonad maturation and reproduction. Stunted gonad development and a lack of spawning have previously been observed at salinities below 5 (Loosanoff, 1948). Delayed or arrested gametogenesis may increase the ability of an oyster to tolerate the stress associated with low salinity exposure because energy can be allocated to somatic maintenance and cellular repair rather than gametogenesis.

Another major difference between this study and previous challenges was the size of oysters used, which is known to affect overall mortality (Southworth et al., 2017) and the timing of mortality in low salinity laboratory-based challenges (e.g. McCarty et al., 2022, 2020). While all challenges have used VIMS ABC low salinity lines, the oysters used in this study (short-term and long-term challenge) were smaller and younger (< 40 mm, 1-yr old) compared to the adult individuals (>80 mm) used previously (McCarty et al., 2020, 2022). Mortality peaked after 6 weeks at 27°C in the short-term challenge (experimental conditions more comparable to previous low salinity challenges: constant temperature of 27°C and 1-2 month exposure duration), while peak mortality (experimental day with the most mortality) occurred 10-12 days after exposure using >80 mm adults in previous studies (McCarty et al., 2020, 2022). The delay in mortality observed during the short-term challenge in this study suggests that smaller and younger oysters (< 40 mm, 1-yr old) are more tolerant to a salinity <3 and temperature 27°C than larger oysters (> 80mm, 2 and 3-yrs old; McCarty et al., 2020, 2022). These results support previous literature suggesting that mortality at specific temperatures and salinities is size-class dependent, where smaller oysters have a higher tolerance to low salinity and high temperature compared to larger

oysters (> 75 mm) (La Peyre et al., 2013; Lowe et al., 2017; McCarty et al., 2020; Rybovich et al., 2016). Smaller oysters are suggested to be more tolerant of stressful conditions because maintenance costs scale with body volume, where a larger individual requires more energy to maintain somatic and gonadal function (Kooijman, 2010).

It is important to investigate stage-specific (e.g. spat/seed, juvenile, adult) correlations with extreme low salinity survival as they will impact the design of breeding programs focusing on this trait. For bivalve species, growth and disease-resistant traits are typically investigated at a single life stage (Dou et al., 2016; Gutierrez et al., 2020, 2018; van Sang et al., 2019; Vu et al., 2021; Wang et al., 2018). However, genetic correlations for growth-related traits measured at 1.5 and 2.5 years old were high (total weight, width index, height index were all > 0.96, meat yield > 0.8; Allen et al., 2021), and correlations were high but slightly lower for survival in a salinity of 6 - 15 (r_G = 0.72; Allen et al., 2021) in the eastern oyster. This suggests, at least for these yield traits, that measurements can be made at an earlier stage and still be predictive of performance later in grow-out. This is useful from an application standpoint, where testing younger individuals may be more convenient (i.e. smaller sizes, smaller experimental setup, less husbandry burden). For low salinity survival, a progeny test using adult oysters will take a shorter period of time to achieve measurable mortality (> 80 mm, 2-4 weeks; McCarty et al., 2020, 2022), but maintaining animals until, at least, 2 years old before testing can be costly from a husbandry standpoint.

4.2 Clearance experiment results and implications for low salinity tolerance

Clearance experiments revealed differences among families in the capacity to remove algae under low salinity stress, though these differences fell just below the 0.05 threshold for

642 significance. In general, families that depleted the available algae more quickly (i.e. higher R_{max},
643 higher CR_{avg}, and lower D_{50}; blue lines Figure 5) were more tolerant of extreme low salinity
644 conditions (i.e. higher survival; Figure 6). In contrast, individuals from families with low
645 cumulative survival in the challenge removed a smaller portion of the initial concentration of
646 algae at lower rates (i.e. lower R_{max}, lower CR_{avg}, and higher D_{50}; black lines Figure 5).
647 Additionally, a significant difference in the three clearance metrics was observed between
648 oysters that survived (N = 143) compared to those that died (N= 35) after being returned to the
649 challenge. This provides additional support that removing algae more quickly (i.e. larger CR_{avg}
650 and R_{max}, smaller D_{50}) may be associated with enhanced low salinity tolerance. While this work
651 is preliminary in nature, it is one of the only studies to demonstrate algal removal (clearance)
652 over 24 hours under such extreme low salinity conditions and contributes to our understanding of
653 how oysters cope physiologically under prolonged exposure.
654 The weak associations between the clearance metrics and challenge survival ($R^2 = 0.05 –$
655 0.10; Figure 6A-C) could be due to the relatively small number of individuals examined (N =
656 178), or the relatively small number of individuals sampled per family on a given sampling day
657 (N= 3-5). There was also high individual variation within each family, which could have also
658 reduced the significance of the relationships. Clearance rates are influenced by many different
659 factors and have proven to be highly variable in shellfish (Cranford et al., 2011, 2005; Grizzle et
660 al., 2008; Li et al., 2012), making it inherently difficult to measure and quantify. However, the
661 two metrics derived from the LOESS curves (time to deplete 50% of the starting algae and
662 maximum removal rate) appear to capture differences in the sigmoidal trends among families
663 better than the standard clearance rate metric and may be useful in future studies. Importantly,
664 we also note that individuals sampled on multiple days, and at slightly different temperatures

(20.7°C - 24.7°C), had consistent performance in clearance metrics (no significant differences in clearance metrics across days), indicating that similar measurements may be conducted across multiple days throughout an experiment to include more individuals and families, although this will need to be repeated with higher replication. Overall, future experiments are needed with larger sample sizes and more replicates per family to better examine the relationship between clearance metrics at low salinity and survival at low salinity. Nevertheless, this is one of the few studies to characterize these feeding metrics and active clearance at such low salinities, and results showed clear differences among control oysters (held at ambient) versus low salinity exposed oysters. Future work determining the physical and physiological traits responsible for differences in survival, such as feeding/clearance, will help to better understand how oysters deal with this extreme stress, and may provide additional traits for future selective breeding of extreme low salinity tolerance.

4.3 Implications of the recovery experiment

After being returned to ambient conditions for ~1 month (salinity 13), all families displayed similar growth rate, wet weight gain, and mortality regardless of survival in the long-term low salinity exposure. Growth rate for all families mirrored that of control individuals (held at ambient salinity) during the 1-month recovery experiment (Figure 7B), suggesting that families are able to resume normal growth once moved back to ambient conditions (higher salinity). From an application standpoint, this finding is quite useful. If field-deployed low salinity-tolerant oysters experience a prolonged low-salinity exposure event, a farm may experience a delay in getting oysters to market size due to reduced growth, but mortality is expected to be low upon return to more typical salinity conditions, and these oysters should

resume normal growth when salinity increases. While the recovery of all families after extreme low salinity exposure is encouraging, it is important to note that we did not investigate recovery for the worst performing families (bottom 10% of families, average long-term challenge mortality of 50%). However, low surviving families in the challenge would be unlikely candidates for a breeding program. Potential carry-over effects of low salinity exposure, such as lower survival during the winter season or lower growth during the next growing season (i.e. long-standing effects), are worth investigating.

5. Conclusion

This work, alongside previous experimental challenges (McCarty et al., 2022, 2020), provides insight into the effect of temperature, exposure duration, and size/age on eastern oyster mortality during extreme low salinity (< 3) events. To date, the results of all five challenge experiments (McCarty et al., 2022, 2020, and this study) indicate that the deployment of a short-term (30-60 day) challenge at a constant temperature (27°C) and salinity (< 3) produces a reliable test of progeny survival, a phenotype useful for extreme low salinity breeding that would be practical to implement in a breeding program. While preliminary, the clearance experiments provide novel data that may be correlated to physiological status or survival probability under salinity stress. Based on our recovery experiment results, oysters that are highly tolerant of extreme low salinity conditions can also be expected to grow as well as control or non-tolerant oysters under typical oligohaline conditions, suggesting no real tradeoff, at least in the short term, between extreme low salinity survival and normal (ambient) growth and survival. The resumption of apparently normal growth for all families tested following long-term exposure to low salinity (< 3) is a promising result and suggests that farms deploying low salinity (< 3 ppt)

tolerant oysters as a proportion of their crop could sustain production in areas that frequently experience periods of extreme low salinity.

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References

Allen, S.K., Small, J.M., Kube, P.D., 2021. Genetic parameters for *Crassostrea virginica* and their application to family-based breeding in the mid-Atlantic, USA. Aquaculture 538, 1–12. https://doi.org/10.1016/j.aquaculture.2021.736578

734 Andrews, J.D., 1964. Oyster mortality studies in Virginia. IV. MSX in James River public seed
735 beds. Proceedings of the National Shellfisheries Association 53, 65–84.
736 Andrews, J.D., Quayle, D.B., Haven, D., 1959. Fresh-water kill of oysters (*Crassostrea*
737 *virginica*) in James River, Virginia, 1958. Proceedings of the National Shellfisheries
738 Association 49, 29–49.
739 Bayne, B.L., 2017. Chapter 5: Feeding, in: Biology of Oysters. Academic Press, pp. 209–329.
740 Beaven, G.F., 1946. Effect of Susquehanna River stream flow on Chesapeake Bay salinities and
741 history of past oyster mortalities on upper bay bars. Chesapeake Biological Laboratory
742 Contribution 1–11.
743 Brammer, A.J., Rodriguez del Rey, Z., Spalding, E.A., Poirrier, M.A., 2007. Effects of the 1997
744 Bonnet Carré Spillway opening on infaunal macroinvertebrates in Lake Pontchartrain,
745 Louisiana. J Coast Res 23, 1292. https://doi.org/10.2112/05-0571.1
746 Brown, K.M., George, G.J., Peterson, G.W., Thompson, B.A., Cowan, J.H., 2008. Oyster
747 predation by black drum varies spatially and seasonally. Estuaries and Coasts 31, 597–604.
748 https://doi.org/10.1007/s12237-008-9045-8
749 Burreson, E., Ragone Calvo, L., 1996. Epizootiology of *Perkinsus marinus* disease of oysters in
750 the Chesapeake Bay with emphasis on data since 1985. J Shellfish Res 15, 17–34.
751 Butler, D.G., Cullis, B.R., Gilmour, A.R., Gogel, B.J., Thompson, R., 2017. ASReml-R
752 Reference Manual Version 4. ASReml-R Reference Manual 176.
753 Butler, P.A., 1952. Growth and mortality rates in sibling and unrelated oyster populations.
754 Proceedings of the Gulf and Caribbean Fisheries Institute 4, 71.

Butler, P.A., 1949. An investigation of oyster producing areas in Louisiana and Mississippi damaged by flood waters in 1945, United States Fish and Wildlife Service, Special Scientific Report: Fisheries No.8. Washington, D.C.

Cheng, B.S., Bible, J.M., Chang, A.L., Ferner, M.C., Wasson, K., Zabin, C.J., Latta, M., Deck, A., Todgham, A.E., Grosholz, E.D., 2015. Testing local and global stressor impacts on a coastal foundation species using an ecologically realistic framework. Glob Chang Biol 21, 2488–2499. https://doi.org/10.1111/gcb.12895

Core Development Team, R., 2020. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

Cornwell, J.C., Rose, J., Kellogg, M.L., Luckenbach, M.W., Bricker, S.B., Paynter, K.T., Moore, C., Parker, M., Sanford, L., Wolinski, B., Lacatell, A., Fegley, L., Hudson, K., 2016. Panel recommendations on the oyster BMP nutrient and suspended sediment reduction effectiveness determination decision framework and nitrogen and phosphorus assimilation in oyster tissue reduction effectiveness for oyster aquaculture practices. Oyster BMP Expert Panel First Incremental Report.

Coughlan, J., 1969. The estimation of filtering rate from the clearance of suspensions. Mar Biol 2, 356–358. https://doi.org/10.1007/BF00355716

Craig, A., Powell, E.N., Fay, R.R., Brooks, J.M., Craig, A., Powell, E.N., Fay, R.R., Brooks, J.M., 1989. Distribution of *Perkinsus marinus* in Gulf Coast Oyster Populations. Estuaries 12, 82–91.

Cranford, P.J., Armsworthy, S.L., Mikkelsen, O.A., Milligan, T.G., 2005. Food acquisition responses of the suspension-feeding bivalve *Placopecten magellanicus* to the flocculation

and settlement of a phytoplankton bloom. J Exp Mar Biol Ecol 326, 128–143. https://doi.org/10.1016/j.jembe.2005.05.012

Cranford, P.J., Strohmeier, T., Filgueira, R., Strand, Ø., 2016. Potential methodological influences on the determination of particle retention efficiency by suspension feeders: *Mytilus edulis* and *Ciona intestinalis*. Aquat Biol 25, 61–73. https://doi.org/10.3354/ab00660

Cranford, P.J., Ward, J.E., Shumway, S.E., 2011. Bivalve Filter Feeding: Variability and Limits of the Aquaculture Biofilter, in: Shumway, S.E. (Ed.), Shellfish Aquaculture and the Environment. John Wiley & Sons, Inc., pp. 81–124. https://doi.org/10.1002/9780470960967.ch4

Dou, J., Li, X., Fu, Q., Jiao, W., Li, Y., Li, T., Wang, Y., Hu, X., Wang, S., Bao, Z., 2016. Evaluation of the 2b-RAD method for genomic selection in scallop breeding. Sci Rep 6, 1–11. https://doi.org/10.1038/srep19244

Du, J., Park, K., Jensen, C., Dellapenna, T.M., Zhang, W.G., Shi, Y., 2021. Massive oyster kill in Galveston Bay caused by prolonged low-salinity exposure after Hurricane Harvey. Science of the Total Environment 774, 1–10. https://doi.org/10.1016/j.scitotenv.2021.145132

Engle, J.B., 1946. Commercial aspects of the upper Chesapeake Bay oyster bars in light of the recent oyster mortalities. National Shellfisheries Association 48, 42–46.

Galtsoff, P.A., 1964. The American oyster *Crassostrea virginica* Gmelin. Fishery Bulletin 64, 1–480.

Gledhill, A., James, H., Fairly, A., Kristine, L., Gregory, L., Gledhill, J.H., Barnett, A.N.N.F., Slattery, M., Willett, K.L., Easson, G.L., Otts, S.S., 2020. Mass mortality of the Eastern Oyster *Crassostrea virginica* in the western Mississippi Sound following unprecedented

Mississippi River flooding in 2019. J Shellfish Res 39, 235–244. https://doi.org/10.2983/035.039.0205

Grizzle, R.E., Greene, J.K., Coen, L.D., 2008. Seston removal by natural and constructed intertidal eastern oyster (*Crassostrea virginica*) reefs: A comparison with previous laboratory studies, and the value of in situ methods. Estuaries and Coasts 31, 1208–1220. https://doi.org/10.1007/s12237-008-9098-8

Gunter, G., 1953. The relationship of the Bonnet Carre Spillway to oyster beds in the Mississippi Sound and the Louisiana Marsh with a report on the 1950 opening. Publications of the Institute of Marine Science 21–72.

Gutierrez, A.P., Matika, O., Bean, T.P., Houston, R.D., 2018. Genomic selection for growth traits in Pacific Oyster (*Crassostrea gigas*): potential of low-density marker panels for breeding value prediction. Front Genet 9, 1–9. https://doi.org/10.3389/fgene.2018.00391

Gutierrez, A.P., Symonds, J., King, N., Steiner, K., Bean, T.P., Houston, R.D., 2020. Potential of genomic selection for improvement of resistance to Ostreid Herpes virus in Pacific oyster (*Crassostrea gigas*). Anim Genet 1–9. https://doi.org/10.1101/754473

Harrell, F.E., 2021. Package 'Hmisc.'

Hopkins, S.H., 1962. Distribution of species of *Cliona* (boring sponge) on the Eastern Shore of Virginia in relation to salinity. Chesapeake Science 3, 121–124. https://doi.org/10.2307/1351224

Hudson, K., 2019. Virginia shellfish aquaculture situation and outlook report: results of the 2018 Virginia shellfish aquaculture crop reporting survey, Marine Resource Report No. 2019-8; Virginia Sea Grant VSG-19-03.

822 Kooijman, S.A.L.M., 2010. Dynamic Energy Budget theory for metabolic organisation, 3rd ed.
823 Cambridge University Press, New York.
824 La Peyre, M.K., Eberline, B.S., Soniat, T.M., La Peyre, J.F., 2013. Differences in extreme low
825 salinity timing and duration differentially affect eastern oyster (*Crassostrea virginica*) size
826 class growth and mortality in Breton Sound, LA. Estuar Coast Shelf Sci 135, 146–157.
827 https://doi.org/10.1016/j.ecss.2013.10.001
828 La Peyre, M.K., Geaghan, J., Decossas, G., Peyre, J.F. La, 2016. Analysis of environmental
829 factors influencing salinity patterns, oyster growth, and mortality in lower Breton Sound
830 estuary, Louisiana, using 20 years of data. J Coast Res 32, 519–530.
831 https://doi.org/10.2112/jcoastres-d-15-00146.1
832 Lambert, C., Moal, C., le Moullac, G., Pouvreau, S., 2008. Mortality risk associated with
833 physiological traits of oysters during reproduction, in: Summer Mortality of the Pacific
834 Oyster *Crassostrea Gigas* – The Morest Project. pp. 63–101.
835 Leonhardt, J.M., Casas, S., Supan, J.E., La Peyre, J.F., 2017. Stock assessment for eastern oyster
836 seed production and field grow-out in Louisiana. Aquaculture 466, 9–19.
837 https://doi.org/10.1016/j.aquaculture.2016.09.034
838 Li, Y., Meseck, S.L., Dixon, M.S., Rivara, K., Wikfors, G.H., 2012. Temporal variability in
839 phytoplankton removal by a commercial, suspended eastern oyster nursery and effects on
840 local plankton dynamics. J Shellfish Res 31(4), 1077-1089
841 Li, Y., Qin, J.G., Abbott, C.A., Li, X., Benkendorff, K., 2007. Synergistic impacts of heat shock
842 and spawning on the physiology and immune health of *Crassostrea gigas*: An explanation
843 for summer mortality in Pacific oysters. Am J Physiol Regul Integr Comp Physiol 293,
844 2353–2362. https://doi.org/10.1152/ajpregu.00463.2007

Loosanoff, V.L., 1952. Behavior of oysters in water of low salinity. Proceedings of the National Shellfisheries Association 43, 135–151.

Loosanoff, V.L., 1948. Gonad development and spawning of oysters (*O. virginica*) in low salinities. Anat Rec 101.

Lowe, M.R., Sehlinger, T., Soniat, T.M., Peyre, M.K. La, 2017. Interactive effects of water temperature and salinity on growth and mortality of eastern oysters, *Crassostrea virginica*: a meta-analysis using 40 years of monitoring data. J Shellfish Res 36, 683–697. https://doi.org/10.2983/035.036.0318

MacKenzie, C.L., 1970. Causes of oyster spat mortality, conditions of oyster setting beds, and recommendations for oyster bed management. Proceedings of the National Shellfisheries Association 60, 1–8.

Mann, R.L., Evans, D.A., 1998. Estimation of oyster, *Crassostrea Virginica*, standing stock, larval production and advective loss in relation to observed recruitment in the James River, Virginia. J Shellfish Res 17, 239–253.

Manzi, J.J., 1970. Combined effects of salinity and temperature on the feeding, reproductive, and survival rates of *Eupleura caudata* (Say) and *Urosalpinx cinerea* (Say) (Prosobranchia: Muricidae). Biological Bulletin 138, 35–46.

Marascuilo, L.A., McSweeney, M., 1967. Nonparametric post hoc comparisons for trend. Psychol Bull 67, 401–412.

McCarty, A.J., Allen, S.K., Plough, L. v., 2022. Genome-wide analysis of acute low salinity tolerance in the eastern oyster *Crassostrea virginica* and potential of genomic selection for trait improvement. G3: Genes, Genomes, Genetics 12. https://doi.org/10.1093/G3JOURNAL/JKAB368

McCarty, A.J., McFarland, K., Small, J., Allen, S.K., Plough, L. v., 2020. Heritability of acute low salinity survival in the Eastern oyster (*Crassostrea virginica*). Aquaculture 529, 735649. https://doi.org/10.1016/j.aquaculture.2020.735649

McFarland, K., Donaghy, L., Volety, A.K., 2013. Effect of acute salinity changes on hemolymph osmolality and clearance rate of the non-native mussel, *Perna viridis*, and the native oyster, *Crassostrea virginica*, in Southwest Florida. Aquat Invasions 8, 299–310. https://doi.org/10.3391/ai.2013.8.3.06

McFarland, K., Rumbold, D., Loh, A.N., Haynes, L., Tolley, S.G., Gorman, P., Welch, B., Goodman, P., Barnes, T.K., Doering, P.H., Soudant, P., Volety, A.K., 2022. Effects of freshwater release on oyster reef density, reproduction, and disease in a highly modified estuary. Environ Monit Assess 194. https://doi.org/10.1007/s10661-021-09489-x

Melancon, E.Jr., 1990. Environmental and economic influences on the oyster fishery of Lower Barataria Bay, Louisiana. Louisiana State University 1–173.

Munroe, D., Tabatabai, A., Burt, I., Bushek, D., Powell, E.N., Wilkin, J., 2013. Oyster mortality in Delaware Bay: Impacts and recovery from Hurricane Irene and Tropical Storm Lee. Estuar Coast Shelf Sci 135, 209–219. https://doi.org/10.1016/j.ecss.2013.10.011

Paynter, K.T., Dimichele, L., 1990. Growth of tray-cultured oysters (*Carssostrea virginica* Gmelin) in Chesapeake Bay. Aquaculture 87, 289–297. https://doi.org/10.1016/0044-8486(90)90066-V

Riisgård, H., 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Mar Ecol Prog Ser 45, 217–223. https://doi.org/10.3354/meps045217

889 Rybovich, M., Peyre, M.K. La, Hall, S.G., Peyre, J.F. La, 2016. Increased temperatures
890 combined with lowered salinities differentially impact oyster size class growth and
891 mortality. J Shellfish Res 35, 101–113. https://doi.org/10.2983/035.035.0112
892 Samain, J.-F., McCombie, H., 2008. Summer mortality of Pacific Oyster *Crassostrea gigas*.
893 Versailles: Editions Quae, The Morest Project.
894 Schubel, J.R., Pritchard, D.W., 1986. Responses of upper Chesapeake Bay to variations in
895 discharge of the Susquehanna River. Estuaries 9, 236–249. https://doi.org/10.2307/1352096
896 Shumway, S.E., 1996. Natural Environmental Factors, In V. S. Kennedy, R. I. Newell, & A. F.
897 Eble (Eds.), *The Eastern Oyster Crassostrea virginica*. (pp. 467-511) Maryland Sea Grant,
898 College Park, MD.
899 Southworth, M., Harding, J.M., Wesson, J.A., Mann, R., 2010. Oyster (*Crassostrea virginica*,
900 gmelin 1791) population dynamics on public reefs in the great Wicomico River, Virginia,
901 USA. J Shellfish Res 29, 271–290. https://doi.org/10.2983/035.029.0202
902 Southworth, M., Long, M.C., Mann, R., 2017. Oyster (*Crassostrea virginica* [Gmelin, 1791])
903 mortality at prolonged exposures to high temperature and low salinity. J Shellfish Res 36,
904 335–340. https://doi.org/10.2983/035.036.0205
905 Tarnowski, M., 2020. Maryland Oyster Population Status Report 2019 Fall Survey. Annapolis.
906 van Sang, V., Knibb, W., Hong Ngoc, N.T., van In, V., O'Connor, W., Dove, M., Nguyen, N.H.,
907 2019. First breeding program of the Portuguese oyster *Crassostrea angulata* demonstrated
908 significant selection response in traits of economic importance. Aquaculture 734664.
909 https://doi.org/10.1016/j.aquaculture.2019.734664
910 van Senten, J., Engle, C., Parker, M., Webster, D., 2019. Analysis of the economic benefits of
911 the Maryland shellfish aquaculture industry.

912 Vu, S. v, Gondro, C., Nguyen, N.T.H., Gilmour, A.R., Tearle, R., Knibb, W., Dove, M., Vu, I.
913 van, Khuong, L.D., Connor, W.O., 2021. Prediction accuracies of genomic selection for
914 nine commercially important traits in the Portuguese Oyster (*Crassostrea angulata*) using
915 DArT-Seq Technology. Genes (Basel) 12, 1–14.
916 Wang, Y., Sun, G., Zeng, Q., Chen, Z., Hu, X., Li, H., Wang, S., Bao, Z., 2018. Predicting
917 Growth Traits with Genomic Selection Methods in Zhikong Scallop (*Chlamys farreri*).
918 Marine Biotechnology 20, 769–779. https://doi.org/10.1007/s10126-018-9847-z
919 Wilson, A.J., Réale, D., Clements, M.N., Morrissey, M.M., Postma, E., Walling, C.A., Kruuk,
920 L.E.B., Nussey, D.H., 2010. An ecologist's guide to the animal model. Journal of Animal
921 Ecology 79, 13–26. https://doi.org/10.1111/j.1365-2656.2009.01639.x
922