ABSTRACT

Title of Dissertation:	EXAMINING THE GENETIC BASIS AND PHYSIOLOGY OF SURVIVAL IN EXTREME LOW SALINITY TO IMPROVE AQUACULTURE OF THE EASTERN OYSTER *Crassostrea virginica*
	Alexandra Jan McCarty, Doctor of Philosophy, 2022
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The eastern oyster, *Crassostrea virginica*, is an important aquaculture species and supports a growing industry along the east coast of the United States. However, increases in freshwater from storm events and intentional diversions can expose coastal aquaculture operations to extreme low salinity (< 5), resulting in reduced productivity and mortality. The primary objectives of this dissertation were to investigate the biology and genetic basis of low salinity tolerance to improve eastern oyster aquaculture. In *Chapter 2*, I developed and conducted a series of extreme low salinity (2.5) challenges to estimate the quantitative genetic parameters of low salinity survival. A moderate narrow-sense heritability was estimated for challenge survival, $h^2 \cong 0.4$. In addition, osmolality of hemolymph collected from oysters during the first week of the challenge suggest that all individuals conformed to the surrounding low salinity regardless of challenge survival. In *Chapter 3*, I performed additional low salinity challenges to assess the importance of challenge duration (2 or 6 months) and temperature (chronic or fluctuating) on low

salinity survival. I also investigated algae removal during the chronic challenge to better understand oyster response during low salinity stress. Phenotypic (r_S = 0.89) and genetic (r_G = 0.81) correlations between family mortality were high across the two challenges, indicating that a 30-day exposure at a constant low salinity (2.5) and temperature (27°C) is a sufficient progeny test for low salinity survival. Modest associations between algae removal metrics and survival in extreme low salinity indicate that individual feeding ability may relate to differential low salinity survival. Lastly, in *Chapter 4*, I performed genome mapping to investigate the genomic architecture of low salinity survival. Quantitative trait locus mapping and linkage disequilibrium analysis revealed a significant region on eastern oyster chromosome 1 and 7. Genomic prediction accuracies for survival and day to death in extreme low salinity were moderate and encouraging, 0.49 – 0.57. The results from my dissertation characterize the genetic basis of survival during low salinity events and support the incorporation of this trait into breeding efforts to improve production and enhance the resiliency of the aquaculture industry.

EXAMINING THE GENETIC BASIS AND PHYSIOLOGY OF SURVIVAL IN EXTREME LOW SALINITY TO IMPROVE AQUACULTURE OF THE EASTERN OYSTER (*Crassostrea virginica*)

by

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Preface

This dissertation contains three research chapters that were conducted and written in collaboration with my academic advisor, Louis Plough. Chapters 2 and 4 are published in academic journals with myself as the primary author and my academic advisor as a major contributor. Multiple co-authors provided mentorship during the experimental design process, assisted with executing the scientific experiments, and helped when writing and editing the manuscripts. At the end of each chapter, I acknowledge my funding sources and collaborators.

Dedication

To my mother, Pamela McCarty, who was by my side every step of the way.

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Chapter 1: Introduction

Quantitative genetics of selection and animal breeding

For a given environment, nature selects individuals with traits that make them more likely to survive, mature, and reproduce. The theory of natural selection, famously described as 'survival of the fittest', was developed by Charles Darwin when describing physical traits and behavioral patterns of numerous species in the Galapagos Islands and in other areas (Darwin, 1859). Natural selection occurs generation after generation, typically increasing the number of organisms within a population that are better suited for a specific environment due to the presence of desirable traits (Darwin, 1859). If the environment changes, then the selection pressure may also change and populations of organisms may adapt and evolve in a different direction. Artificial selection works in the same manner as natural selection, except humans are acting as the selective agent (Darwin, 1859). Humans first identify traits that are desirable for a particular reason or purpose, and then take action to increase and enhance the frequency of that trait in future generations (methods reviewed in Bourdon, 2000).

Selection shifts the mean measurable phenotypic value for a trait in a population towards a specific value. Selection is successful when there is both variation in the observable phenotype, and when the phenotypic variation is controlled by variations in underlying genetic components (Falconer and Mackay, 1996). For example, dicotyledonous plants vary in the number of cotyledons (embryonic leaves) present on a germinating seed. In 1944, Holtorp bred *Brassica* plants (e.g. cabbage, cauliflower, savoy) for tricotyledony, which is the presence of three cotyledons on the emerging seed (Holtorp, 1944). Holtorp observed increased tricotyledony in subsequent generations after hand-selecting offspring to seed that displayed tricotyledony

(1944). In this case, selection was effective at increasing tricotyledony in future generations, suggesting that tricotyledony in *Brassica* plants is controlled by underlying genetic components.

A trait is heritable if the underlying alleles or genetic variants have an additive effect on the trait and if the trait varies between individuals in a population. Heritable traits are passed from parent to offspring as a collection of genes or alleles. The variation in a phenotypic trait (V_P) in a population can be divided into variance from genotypic effects (V_G) and variance from environmental deviations (V_E; Falconer and Mackay, 1996):

$$V_P = V_G + V_E$$

The variation from genotypic effects is further divided into additive (V_A), dominance (V_D), and epistatic (V_{Epi}) effects (Falconer and Mackay, 1996):

$$V_G = V_A + V_D + V_{Epi}$$

Specifically, narrow-sense heritability (h^2) for a trait in a population is the proportion of phenotypic variance that is attributed to additive genetic variation (Falconer and Mackay, 1996):

$$h^2 = V_A/V_P$$

The additive genetic effect of a trait is the summation of all alleles underlying that particular trait of interest (Falconer and Mackay, 1996). The genetic merit of an individual for a specific trait is referred to as a breeding value, where a larger breeding value suggests higher potential for genetic gain in that individual's offspring (Falconer and Mackay, 1996). A high narrow-sense heritability value for a trait suggests that the breeding value of an individual, or the additive genetic variance of a population, is a good indicator of phenotypic performance (Bourdon, 2000; Falconer and Mackay, 1996). Therefore, all types of selection, natural and artificial, are successful when acting on heritable traits, or traits controlled mainly by additive genetic effects.

When selecting for quantitative traits, or traits controlled by many loci and affected by environmental conditions, one is concerned with the phenotypic co-variation among relatives (degree of resemblance; Falconer and Mackay, 1996). The phenotypic co-variation among related groups, which is a proportion of the total phenotypic variation, can be used to determine the additive genetic variance of a trait, and thus determine the narrow-sense heritability of the trait (Falconer and Mackay, 1996). In an experimental setting, groups of individuals with a known relationship structure (e.g. parents and offspring, half-siblings, full-siblings, etc.) are used because the phenotypic covariance is made up of established proportions of the various variance components (mentioned above). For example, offspring are expected to share 50% of their genetic makeup with each parent (coefficient of relationship = 0.5), full siblings share 50%, half-siblings share 25%, etc. (Falconer and Mackay, 1996). If the experimental group comprises e.g. parents and offspring, then the additive genetic variance of a trait is equal to 2 times the phenotypic co-variance between the offspring and both parents (phenotypic covariance = $\frac{1}{2}V_{A;}$ Falconer and Mackay, 1996). In practice, the mean phenotypic performance of offspring is regressed on the mean phenotypic value of one or both parents, and the slope of the regression line is the estimated narrow-sense heritability (slope is multiplied by two if only one parent is used because it is twice the regression on single parents; Falconer and Mackay, 1996). Estimating the additive genetic variance and heritability for siblings (half or full) is slightly more complex and requires the partitioning of the phenotypic variance into three components: variance between progeny of different fathers, variance between progeny of different mothers mated to the same father, and variance between offspring of the same mother (Falconer and Mackay, 1996). An analysis of variance (ANOVA) framework can be used to estimate each of these variance components, and then the narrow-sense heritability can be estimated mathematically

based on the family structure (Falconer and Mackay, 1996). Individuals are then selected for mating based on their individual or family mean phenotypic performance, known as individual or family-based breeding (reviewed in Bourdon, 2000).

In practice, determining the phenotypic covariance between relatives is difficult. These estimations are difficult because there are many additional sources of variation underlying a phenotypic trait (e.g. environmental variance), many experiments have more complex relationship structure than the traditional textbook examples (parent-offspring, half-sibling, etc.), and many experiments do not lend themselves well to these straightforward calculations (i.e. uneven family sizes, uneven replicates, etc.). Hence, the best linear unbiased predictor approach (BLUP) was developed (reviewed in Lynch and Walsh, 1998). BLUP allows for the estimation of random effect components, which permits the incorporation of additional sources of variation inherent to experimental design (e.g. blocking structure, replicate structure, etc.). Most importantly, BLUP allows for the incorporation of a relationship matrix, either estimated from a pedigree or from genomic information, as a random effect. In ecology, these linear models are referred to as the animal model (reviewed in Kruuk, 2004; methods described in Lynch and Walsh, 1998).

To summarize, the known, or determinable, relationship structure between groups of individuals allows for the estimation of the phenotypic covariance (degree of resemblance). From this, the additive genetic variance of a trait can be estimated, and consequently, the narrow-sense heritability of a trait. While this can be carried out using regression and analysis of variance (ANOVA) for simple cases, the animal model (i.e. BLUP implemented in either Frequentist or Bayesian approaches) allows for the incorporation of complex relationship structures, additional sources of variation, and also accounts for differences in family size and

replicates. In the practice of selective breeding, traits with high-narrow sense heritability are good candidates for breeding programs because much of the phenotypic variation is due to additive effects. Additive effects are inherited from parents to offspring as genetic material (alleles and genes) and individuals are chosen for breeding based on their genetic merit, or their collection of alleles and genes controlling that particular trait of interest.

Selective breeding approaches in animal production

In general, the distribution and scale of genetic effects that combine to control a phenotypic character (i.e. genetic architecture of a trait; Hansen, 2006) will dictate which breeding method is most effective. Traditionally, as in Holtorp's experiments, the "best" performers, or individuals displaying the higher than average (desired) phenotype, were mated/reproduced over many generations in hopes of shifting the mean phenotype towards tricotyledony (Holtorp, 1944). Individual and family-based breeding can be effective in making rapid genetic gains, but genetic improvement can be hampered if traits are controlled by many genes or are strongly influenced by environmental effects. Genome-based breeding techniques have many advantages over traditional breeding techniques and individuals can be selected for breeding based on the presence of one to a few major effect genes (marker-assisted selection, MAS) or based on the combined effect of genotypes across the genome (genomic selection, GS).

Traditional breeding approaches select individuals based on phenotypic performance. In individual or mass selection, individuals are chosen for selection based on their phenotype (Fjalestad, 2005). Family-based selection is similar, but selection is based on the mean phenotypic performance for a family of individuals (Fjalestad, 2005). Selection based on individual or family-based performance has been successful for various aquaculture species

(reviewed in Fjalestad, 2005), but has limitations. For individual selection, traits have to be measured on the breeding individual while they are alive, which is difficult for traits such as disease resistance and meat quality (Fjalestad, 2005). In practice, keeping individuals and families separate and organized before breeding is difficult and requires large rearing facilities, especially considering the large number of individuals required for accurate estimations and to avoid substantial inbreeding (Fjalestad, 2005). It is also difficult to keep environmental conditions similar across individuals or families, which can confound the phenotypic trait measured (Fjalestad, 2005). Using molecular markers to determine relationship eliminates the necessity of keeping individuals separate, subsequently eliminating some of the confounding environmental effects from the experimental design (e.g. blocking structure, differences in experimental tanks; Gjerde, 2005; Hollenbeck and Johnston, 2018). In addition, molecular markers allow for the precise computation of relationship structure versus the percentage assumed based on pedigree relationship (e.g. full-siblings share 50% of DNA; Gjerde, 2005), producing more accurate estimations of genetic variance components and narrow-sense heritability.

In marker-assisted selection (MAS), individuals are chosen for breeding depending on the presence of a major genomic region underlying a trait of interest. Genomic regions underlying phenotypes of interest can be identified by performing quantitative-trait locus (QTL) mapping or genome-wide association studies (GWAS; e.g. Hollenbeck and Johnston, 2018; Lynch and Walsh, 1998; Zenger et al., 2019). In short, both methods test for associations between genotypes at molecular makers (e.g. single nucleotide polymorphisms (SNPs), microsatellites, etc.) and phenotypes. These two techniques take advantage of genetic linkage, where genes located more closely to one another on a chromosome are more likely to be inherited together (no

recombination between them) during reproduction, and linkage disequilibrium, which is the probability that two alleles are more likely to occur together than by chance within a population (Falconer and Mackay, 1996). If enough genomic markers are interrogated across the genome (e.g. genotyping of high-density, genome-wide SNP markers), while they may not themselves have any direct impact on a trait, their linkage to the actual genomic regions/genes that do affect or underlie a trait can be detected (Lynch and Walsh, 1998). Thus, the detection of a QTL or discovery of a significant association for a particular (random) genetic marker or genomic position (SNP) indicates linkage to the actual gene that affects the trait (Lynch and Walsh, 1998). In practice, an organism would be chosen for breeding depending on the presence of a particular allele at the identified molecular region. For MAS to be effective, there must be relatively few major effect QTL underlying a trait of interest, which is rarely the case, especially for quantitative traits (reviewed in Houston et al., 2020; Zenger et al., 2019).

In genomic selection (GS), individuals are chosen for breeding based on their genomic-derived breeding value (GEBV). The methodology of genomic selection (GS) is based on the process of selecting an individual for breeding (i.e. for producing the next generation) based on the combined effect of all relevant genotypes across its genome (Meuwissen et al., 2001). In theory, GS captures all the genetic variance for a quantitative trait by assuming genotyped molecular markers are linked to actual QTL (Meuwissen et al., 2001), similar to QTL mapping. Individuals with larger GEBVs are chosen for breeding because they are more superior for that particular trait of interest (VanRaden, 2008). Similar to GWAS approaches, GS uses linear and non-linear models to investigate associations between genome-wide genotypes and phenotypes. Validation approaches can be used to generate and assess the accuracy of prediction models,

where models for traits with high accuracy suggest that an individual's genome-wide genotypes are a good indicator of their predicted phenotype.

GS can substantially increase the rate of genetic gain by incorporating all loci, even those of very small effect, into the breeding decision (Houston et al., 2020; Ødegård et al., 2014; Zenger et al., 2019). Incorporation of information across all loci results in better estimation of relationships between individuals (Habier et al., 2007; Vallejo et al., 2017), and GS has been shown to outperform traditional breeding where relationships are derived from pedigree information only (PBLUP; e.g. Gutierrez et al., 2020, 2018; Zenger et al., 2019). In GS, the generation interval, or the average age of individuals when bred, can be reduced by selecting candidates earlier in life based on their GEBV (e.g. Campos-Montes et al., 2013; Castillo-Juárez et al., 2015), further increasing the rate of genetic gain and freeing up infrastructure which would be used if rearing animals longer. Infrastructure requirements could be decreased further by changing to a multi-family breeding scheme where families are reared in a communal space (Fernández et al., 2014). Additionally, choosing broodstock for spawning based on GEBV and known coancestry makes it easier to control and minimize the rate of inbreeding (Daetwyler et al., 2007; Dekkers, 2007; e.g. Vandeputte and Haffray, 2014). This is particularly important for aquaculture species that are highly fecund, have small effective population sizes, and for which relatively few parents could contribute the majority of gametes to offspring in a spawning event (very high variance in reproductive success; Hedgecock and Pudovkin, 2011; reviewed in Hollenbeck and Johnston, 2018). Lastly, further genetic gains in GS, compared to traditional breeding approaches mentioned previously, can be achieved by managing genotype-by-environment interactions and incorporating dominance and epistatic effects into the breeding decision (Dupont-Nivet et al., 2008; Nilsson et al., 2016; Zenger et al., 2019). Genomic-based

breeding has been efficient and effective for the aquaculture species on which it has been tested (examples reviewed in both Houston et al., 2020; Zenger et al., 2019), but genomic resources are limited and genotyping can be costly for non-model species.

Recent advances of genome-enabled breeding in aquaculture species

Reference genomes are now available for select aquaculture species. Over 20 species of finfish have their genomes sequenced, such as Atlantic and Coho Salmon, Nile tilapia, Rainbow trout, Grass carp, and Snakehead (Houston et al., 2020). Reference genomes exist for only six mollusc species (Hollenbeck and Johnston, 2018; Houston et al., 2020), two of which are the Pacific (*Crassostrea gigas*) and eastern (*Crassostrea virginica*) oysters (Gómez-Chiarri et al., 2015; Wang et al., 2012). Advances in sequencing technologies have reduced the cost of genotyping for non-model organisms (Hollenbeck and Johnston, 2018; Houston et al., 2020; Zenger et al., 2019). Reference genomes allow for the identification of causative genetic variants (QTL mapping and GWAS analysis), epigenetic markers for certain environmental conditions, and comparative genomic analysis to help determine function of certain variants (reviewed in Houston et al., 2020), all of which help hone in on certain genes and pathways worthy of additional investigation. Major genes have been successfully incorporated into MAS breeding for disease-resistance in Japanese flounder (Fuji et al., 2007), Atlantic salmon (Houston et al., 2008; Moen et al., 2015, 2009), and Rainbow trout (Liu et al., 2018). However, examples of successful MAS in aquaculture are limited and are most often only useful for discrete traits (e.g. disease-resistance).

Genotyping arrays (i.e. SNP arrays) have improved genomic selection for aquaculture species by increasing prediction accuracies. Single nucleotide polymorphism (SNP) arrays allow for the genotyping of individuals at 1,000s – 10,000s (medium density) or > 100,000s (high-

density) genome-wide SNPs for a relatively low cost (reviewed in Hollenbeck and Johnston, 2018; Houston et al., 2020; Zenger et al., 2019). In addition, analysis across multiple aquaculture species have suggested that as few as 1,000 molecular markers (SNPs) can be genotyped before prediction accuracy decreases (e.g. Kriaridou et al., 2020; reviewed in Zenger et al., 2019), making GS possible for programs with limited monetary funds. However, genotyping arrays exist for only a handful of aquaculture species (e.g. Atlantic salmon, Rainbow trout, Nile tilapia, Catfish, Whiteleg shrimp, Giant tiger prawn; reviewed in Hollenbeck and Johnston, 2018; Houston et al., 2020; Zenger et al., 2019) and only one shellfish species (Pacific oyster; Gutierrez et al., 2017; Qi et al., 2017). Implementation of GS is currently limited to a few economically important species (Rainbow trout, Atlantic salmon, and the Tasmanian Atlantic Salmon strain; reviewed in Houston et al., 2020; Zenger et al., 2019). However, high accuracy values have been reported for numerous aquaculture species and traits (reviewed in Houston et al., 2020; Zenger et al., 2019), offering promising support for the use of GS in future aquaculture breeding.

Development of genomic resources (e.g. organism reference genomes, genotyping SNP arrays), combined with decreases in the cost of sequencing and genotyping, have increased the feasibility of genome-based breeding approaches in aquaculture species (reviewed by Hollenbeck and Johnston, 2018; Houston et al., 2020; Zenger et al., 2019). Finfish have the most abundant genomic resources of all aquaculture species, but an analysis of recent peer-reviewed literature revealed a stable increase in omic-related mollusc papers from 2009 – 2019, with most papers pertaining to resources being applied to bivalves (Gomes-dos-Santos et al., 2020). Given the current availability, or ongoing development, of these genomic resources for oysters, the

future looks bright for targeted genome-based breeding for traits that will improve aquaculture production in the face of emerging environmental stressors.

Global shellfish aquaculture and the rise of shellfish production and breeding

Aquaculture has out-produced capture fisheries since 2011 (FAO, 2018a) and is the fastest growing sector of food production worldwide (Anderson et al., 2017; FAO, 2018b). Global meat production is primarily dominated by three terrestrial species - pigs, chicken, cattle - while an estimated 543 different species of finfish and shellfish are used in global aquaculture (FAO, 2018a; Houston et al., 2020). Production from shellfish aquaculture, which include species of mollusks and crustaceans (FAO, 2020a), has increased 10-fold from 1985 – 2020, and shellfish are projected to be the most valuable culture group (FAO, 2020a). In general, the biology of shellfish are well understood and culture methods are well documented (Azra et al., 2021). Many traits of shellfish (i.e. highly fecund, short life cycle, high levels of genetic diversity, broadcast spawners, possibility of self-fertilization) make them appealing for production and mass-culture (Azra et al., 2021; Hollenbeck and Johnston, 2018; Houston et al., 2020). Additionally, shellfish supply a large source of protein and other desirable nutrients, making them ideal candidates for consumption (FAO, 2016; Venugopal and Gopakumar, 2017). Aquaculture species, including shellfish, are in the relatively early stages of domestication, resulting in a large potential for genetic improvement (FAO, 2019, 2018; Houston et al., 2020). Therefore, expansion and genetic improvement of desirable traits in shellfish will increase production to help meet consumption demands.

According to the literature, large-scale breeding programs for shellfish are limited. Two family-based breeding programs exist for mussels (blue and green lip mussel), one mass selection program for the Bay scallop, one hybrid mass and family-based selection program for

the Sydney Rock oyster, one family-based (recently changed from mass selection) selection program for the eastern oyster, and five selection programs utilize either mass, family-based, or a combination of the two for the Pacific oyster (Allen et al., 2021; reivewed in Hollenbeck and Johnston, 2018). These programs have largely focused on improving traits related to growth and disease-resistance, while the family-based selection program for the eastern oyster has begun breeding for salinity tolerance (described below) (Allen et al., 2021; reviewed in Hollenbeck and Johnston, 2018). For shellfish species, QTL have been identified for various production traits (e.g. Fang et al., 2021; Guo et al., 2012; reviewed in Hollenbeck and Johnston, 2018; Sauvage et al., 2010; Wang et al., 2016; Zhan et al., 2009; Zhong et al., 2014), and studies have been conducted investigating the applicability of genomic selection for improvement of production traits (reviewed in Houston et al., 2020; Vu et al., 2021; reviewed in Zenger et al., 2019). However, no genome-based breeding programs are currently documented for shellfish.

History and current state of eastern oyster breeding in the United States

The eastern oyster (*Crassostrea virginica)* is a shellfish aquaculture species gaining popularity in the United States. The eastern oyster is a filter-feeding bivalve mollusk that is native to subtidal and intertidal estuaries in the western Atlantic from the Gulf of St. Lawrence in Canada to the Gulf of Mexico, and south through the West Indies along the coasts of Brazil and Argentina (Carriker and Gaffney, 1996; Galtsoff, 1964). Wild fisheries exist for the eastern oyster along the Atlantic coast of the US (e.g. Florida, Gulf of Mexico), but the Chesapeake Bay was once the greatest oyster producing region in the world (Stevenson, 1894). Habitat loss, pollution, overfishing, and the effects of two protozoan parasites, *Perkinsus marinus* and *Haplosporidium nelson*i, have severely decreased annual fishery production by an estimated 99.7% since peak harvests in 1800s (Newell, 1988; Rothschild et al., 1994; Stevenson, 1894;

Wilberg et al., 2011), and production from wild harvest remains <1% of peak fishery (Tarnowski, 2020). In response to decimated wild oyster stocks, the aquaculture industry in the Chesapeake Bay has steadily increased in both the number of leases and in overall production (bushels) (Hudson, 2019; van Senten et al., 2019). Within Maryland, the eastern oyster aquaculture industry (bottom and water column aquaculture) increased by 115% from 2012 – 2018 (van Senten et al., 2019). The Maryland shellfish industry provides many economic benefits to state economies and provides valuable employment opportunities in coastal areas where industry is limited (van Senten et al., 2019).

The Maryland aquaculture industry has been successful, in part, due to previous and ongoing breeding efforts for disease resistance in the eastern oyster. An outbreak of MSX disease, caused by the parasite *Haplosporidium nelsoni,* occurred in Delaware Bay from 1957 – 1959 and resulted in >90% mortality of infected oysters (Haskin and Ford, 1982). In response to the MSX outbreak, the first reported shellfish breeding program began at the Haskin Shellfish Research Laboratory in the 1960s (HSRL) (Haskin and Ford, 1979). Lines selected for MSX-resistance displayed improved survival over successive generations (Ford and Haskin, 1987), and data collected from Delaware Bay revealed little to no detectable MSX and very minimal background mortality (~10% per year) in market size oysters (Guo et al., 2008).

In the 1990s, resistance to Dermo disease, caused by the parasite *Perkinsus marinus*, was incorporated into the breeding program at HSRL. Dermo-disease outbreaks were originally restricted to warmer waters (Chesapeake Bay south to Gulf of Mexico), but a series of warm winters expanded this region north and mortalities as large as 50% were experienced in adult oysters throughout the Chesapeake Bay and the Gulf coast of Florida (Ewart and Ford, 1993; Ford and Smolowitz, 2007). Rutgers developed a new line, the Northeast High Survival (NEH)

line, to incorporate Dermo-resistance into the already developed MSX-resistant lines (Allen, 1993; Guo et al., 2008). In 1997, the Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science (VIMS) was founded with the goal of creating a more organized selective breeding effort for the eastern oyster (Allen et al., 2021). Initially, ABC generated two oyster lines, XB and DEBY, that both displayed moderate resistance to MSX and Dermo (Allen et al., 2021; Dégremont et al., 2015; Frank-Lawale et al., 2014; Peterson et al., 2020; Ragone Calvo et al., 2003).

Polyploidy, or multiple sets of chromosomes, has rapidly improved oyster aquaculture. Triploid oysters have three sets of chromosomes and display sterility, increases in growth (usually 30-40%, but can be as high at 60-80%; Guo et al., 2001), improved meat quality year round, and increased disease resistance (Guo et al., 2008). In 2001, Rutgers produced tetraploid (four sets of chromosomes) eastern oysters using their disease-resistant strains (Guo et al., 2008). Tetraploids (4N) can be mated with diploids (2N) to produce triploids (3N), a technology that was first adapted for use in commercial production of Pacific oysters on the west coast of the US (Guo and Allen, 1994). Superior growth has been observed for triploids grown throughout the mid-Atlantic region (Chesapeake Bay, VA to Katama Bay, MA; e.g. Dégremont et al., 2012; Guo et al., 2008). Growers in Virginia report that they favor triploid seed and >80% of seed planted in 2014 – 2018 were disease-resistant triploids (Hudson, 2019, 2018, 2017; Hudson and Murray, 2016, 2015).

In addition to the major breeding efforts for disease resistance and ploidy manipulation, breeding for yield and other traits have been advanced. The NEH strain from Rutgers (mentioned above) was crossed with the FMF line, a fast growth line developed by the Frank M. Flower's Oyster Company in New York, to create a hybrid oyster (Guo et al., 2008). This hybrid strain,

which inherited the NEH name, is currently available to aquaculture farmers in the northeast US, offering disease resistance (MSX and Dermo) and fast growth in (Debrosse, 2008; Guo et al., 2008). In 1986, the University of Maine began selecting for resistance to the bacterium *Roseovarius crassostreaea*, causing *Rosevarius* Oyster Disease (ROD), in the FMF line (Davis and Barber, 1999). The University of Maine Flowers Select (UMFS) offers fast growth and resistance to ROD for aquaculture in colder waters (Davis and Barber, 1999) where ROD can cause annual mortality in seed oysters ranging from 40-90% in Maine, Massachusetts, and New York (Maloy et al., 2007). Since 2004, the ABC program has been using family-based breeding techniques to generate oyster lines for farmers in the Chesapeake Bay. ABC monitors and selects for economically important traits, such as growth weight, meat yield, and shell shape (Allen et al., 2021). ABC also assesses these traits in both high (ABC HS line, 15 – 23) and low salinity (ABC LS line, salinity 6 – 15) environments, generating salinity-specific seed for various salinity regimes in the Chesapeake Bay (Allen et al., 2021).

Breeding efforts have largely focused on traits that improve performance (e.g. survival, disease-resistance, growth, meat yield), but environmentally-important traits are becoming increasingly more important. Disease-resistant triploid seed is favored by the Virginia aquaculture industry (e.g. Hudson, 2019), but numerous environmental challenges are emerging that have not been addressed. For example, the number and intensity of precipitation events are predicted to increase, along with the occurrence of severe weather storms (Najjar et al., 2010, 2000; Pfahl et al., 2017; St. Laurent et al., 2021). Heavy rainfall events will result in large salinity fluctuations in coastal regions, which will impact production and potentially change algal community dynamics. Water temperature and hypoxia are both predicted to increase in the mid-Atlantic region (Najjar et al., 2010), both of which may further impede aquaculture production.

Research and breeding efforts will need to focus on improving oyster aquaculture during these stressful conditions to improve the resiliency of the industry moving forward.

Effects of low salinity on eastern oyster productivity and aquaculture

Environmental extremes pose continued risks to eastern oyster aquaculture on the east coast of the United States**.** 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). Freshwater pulses are also common in the Gulf of Mexico following flooding, anthropogenic diversions, and the opening of the Bonnet Carré Spillway (Brammer et al., 2007; Butler, 1952a, 1949; Gledhill et al., 2020; Gunter, 1953). While these regions tend to have lower salinities to begin with, 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 (*Crassostrea virginica*) 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 (e.g. La Peyre et al., 2016; Lowe et al., 2017; Rybovich et al., 2016). However, acute drops in salinity (< 5) can be detrimental.

The Chesapeake Bay is a highly variable environment with a strong salinity gradient. The Maryland-portion of the Chesapeake Bay is considered mesohaline, where salinities typically range from 5-14 (Wei, 2019). In Virginia, eastern oyster aquaculture operations face challenges from disease pressure due to higher salinity waters (e.g. *Perkinsus marinus* causing Dermo disease, Burreson and Ragone Calvo, 1996; Bushek et al., 2012; Ragone and Burreson, 1993), while eastern oyster aquaculture in Maryland more commonly faces challenges from low salinity conditions. However, the mid-Atlantic region received substantial amounts of rainfall in 2017 –

2018, which significantly impacted the eastern oyster aquaculture industry throughout the Chesapeake Bay (Hudson, 2019; van Senten et al., 2019). In 2018, the Maryland and Virginia aquaculture industries experienced a 22% and 17% decline, respectively, in total harvest compared to the previous year, which both industries attribute to record low salinity (Hudson, 2019; van Senten et al., 2019).

Low salinity (< 5) negatively impacts many physiological functions in eastern oysters. Studies have shown substantial reductions in valve opening, respiration, clearance, and filtration rates with decreasing salinities (Casas et al., 2018b; Loosanoff, 1952; Shumway and Koehn, 1982), and one study suggests that feeding ceases completely at salinity < 3 (Loosanoff, 1952). Additionally, the negative effects from low salinity are intensified at increased temperatures (La Peyre et al., 2016, 2013; Lavaud et al., 2021; Loosanoff, 1952; Rybovich et al., 2016; Shumway and Koehn, 1982), creating a dual-stressor that is common in the Chesapeake Bay. Ultimately, reductions in vital physiological processes result in decreased growth at low salinities (< 5) (La Peyre et al., 2013; Leonhardt et al., 2017; Loosanoff, 1952; Lowe et al., 2017; Rybovich et al., 2016). Delays in oyster growth and productivity can not only delay harvest time, but can also create a back log of crop that will substantially limit the growing space available for a farmer (Hudson, 2019; van Senten et al., 2019; personal communication w/ MD Extension agent Shannon Hood). In addition to reduced productivity and delays in harvest, extreme low salinity (< 5) has previously resulted in mass mortality events globally (reviewed in Du et al., 2021). Mortality events have been observed following heavy rainfall events in the mid-Atlantic region (Andrews et al., 1959; Engle, 1946; Munroe et al., 2013; Schubel and Pritchard, 1986; Southworth et al., 2017) and in the Gulf of Mexico (Brammer et al., 2007; Butler, 1952b, 1949; Gledhill et al., 2020; Gunter, 1953).

Low salinity regions present challenges for successful aquaculture, but they do provide some advantages. Coastal regions may be preferred for oyster aquaculture compared to deeper waters due to the consistency of oxygen levels year-round (Beckensteiner et al., 2020; Theuerkauf and Lipcius, 2016). Additionally, shallower waters reduce the risk and costs associated with accessing, operating, and managing an oyster lease (Beckensteiner et al., 2020). Most importantly, low salinity environments can provide a refuge from some predators and common oyster diseases, such as MSX and Dermo (e.g. Andrews, 1964; Burreson and Ragone Calvo, 1996; Shumway, 1996). Superior bred oysters could improve production in low salinity areas, thereby expanding the aquaculture industry.

Genetic basis of low salinity tolerance in the eastern oyster

A genetic component underlying oyster salinity performance and survival has been implied many times in the literature (Allen et al., 2021; Leonhardt et al., 2017; Méthé et al., 2015; Newkirk et al., 1977; Southworth et al., 2017). Field and laboratory studies suggest local adaptation to native salinity conditions, where juvenile and adult oysters perform best at salinity levels closest to those at their native site (Andrews et al., 1959; Leonhardt et al., 2017; Marshall et al., 2021; Maynard et al., 2018; Southworth et al., 2017). Differences in expression profiles between oysters exposed to a low (8) and high (16) salinity environment revealed specific genes and processes potentially underlying survival at opposing salinity levels (Eierman and Hare, 2013). Larval oysters are more sensitive and have a narrower tolerance to salinity, but have been shown to perform best in their natal (parental) environments opposed to being cultured at other conditions or locations (Eierman and Hare, 2013; Newkirk et al., 1977; Scharping et al., 2019), suggesting that survival at a given salinity is dependent on life stage and may be influenced by parental condition. However, a recent study found no increases in survival when larvae were

reared in low salinity conditions matching their parental environments, suggesting that transgenerational plasticity contributed from the mothers is not a mechanism for improving low salinity survival in offspring (Griffiths et al., 2021). The ABC program at VIMS recently determined that survival in high salinity (15 – 23) is a different trait than survival in low salinity (6 – 15) (i.e. low genetic correlation between survival in high and low salinity zones, 0.23 ± 0.08; Allen et al., 2021). Understanding the genetics and mechanisms underlying survival in low salinity environments is of increasing importance as heavy rainfall events are predicted to increase with climate change predictions in the United States (Donat et al., 2016; Najjar et al., 2010, 2000; Pfahl et al., 2017; Runkle et al., 2017).

Research focus

Based on previous literature documenting survival differences in oysters at various salinity levels, this dissertation aims to better understand the 1) biology and 2) breeding potential of acute low salinity tolerance in the eastern oyster. To do this, I developed a laboratory-based low salinity challenge experiment with the goal of inducing mortality in an experimentally friendly timeframe. I used the developed challenge experiment and classical quantitative genetic approaches to assess the genetic variance of low salinity survival. I also generated molecular data for individuals exposed to the challenge to investigate the genetic structure and identify potential regions underlying low salinity survival.

In **Chapter 2: Heritability of acute low salinity survival in the Eastern oyster (*Crassostrea virginica*)**, I performed a series of low salinity challenges to estimate the genetic variance and narrow-sense heritability of low salinity survival, as the heritability of a trait is the first step in determining if a trait can be selectively bred for. In **Chapter 3: Evaluating a progeny test and investigating physiology associated with survival in extreme low salinity**

for the eastern oyster *Crassostrea virginica*, I built upon Chapter 2 with additional low salinity challenges to assess the importance of oyster age and size, low salinity exposure duration, and exposure temperature on low salinity survival. I also investigated physiology associated with survival in the experimental challenge, such as growth and algae removal (a proxy for feeding), to better understand oyster response during low salinity stress and to potentially provide additional low salinity phenotypes. In **Chapter 4: Genome-wide analysis of acute low salinity tolerance in the eastern oyster *Crassostrea virginica* and potential of genomic selection for trait improvement**, I generated genome-wide molecular marker data for four experimental families exposed to a low salinity challenge and performed genomic analyses (e.g. quantitative trait locus mapping, genome-wide association studies) to explicitly investigate the genomic architecture of the trait. I also investigated the potential for genomic selection (GS) to accelerate selective gains and improve the efficiency of breeding for low salinity survival. Finally, in the concluding chapter (5), I synthesize the major findings from my research chapters, speculate about the major processes and physiology underlying survival in low salinity conditions, and suggest a preliminary plan for future, genome-enabled low salinity breeding in the eastern oyster.

This dissertation advances knowledge of eastern oyster biology and, physiological response, and contributes genomic information from functional analysis of detected SNPs/QTL during exposure to acute low salinity conditions. This work also advances information relevant to the breeding community by honing a progeny test for assessing tolerance to acute low salinity conditions. Improvement of acute low salinity survival will eventually benefit the eastern oyster aquaculture industry and this work provides the first genomic analysis investigating the potential of genomic selection to improve acute low salinity survival in oysters.

Chapter 2: Heritability of acute low salinity survival in the Eastern oyster (*Crassostrea virginica*)

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Abstract

On the US east coast, Eastern oyster (*Crassostrea virginica*) aquaculture has grown substantially over the last century. As aquaculture expands into previously unexploited areas, there is a need for oyster lines bred for specific environmental conditions. In the Maryland portion of the Chesapeake Bay, and in other coastal areas, salinity tends to be below optimal for typical aquaculture productivity, and these areas frequently experience periods of acute low salinity following heavy rain events. While these areas may hinder oyster growth, they provide a refuge from common oyster diseases. Selective breeding focusing on survival in acute low salinity conditions could expand areas suitable for aquaculture, but the genetic framework underlying this trait is currently unknown. In this study, we estimated the heritability of survival at acute low salinity (< 3) by conducting two month-long low salinity exposure experiments with fifty half-sibling families. Hemolymph osmolality was analyzed during the first eight days of the second exposure experiment to track osmoconformation behavior and investigate potential physiological differences underlying variation in salinity survival among families. There were significant differences in mortality among families for both low salinity exposure experiments, with the majority of mortality occurring between 8 and 14 days of exposure for both experiments. Higher overall mortality was observed during the second experiment (53% in experiment 2 versus 23% in experiment 1), which was conducted during the summer when animals were reproductively active. Narrow-sense heritability estimates for survival were moderate to high using both a Bayesian (MCMCglmm, $h^2 = 0.34$ for experiment 1 and 0.59 for experiment 2) and a likelihood-

based (ASReml-R, $h^2 = 0.4$ for both experiments) approach, and estimates from ASReml-R appeared to be lower compared to MCMCglmm for experiment 2 (ASReml-R $h^2 = 0.4$, MCMCglmm $h^2 = 0.59$). Finally, there were no differences in osmolality among families on either sampling day, but all families remained slightly hyperosmotic after salinity was held static at our desired level (2.5). This study provides the first quantitative genetic analysis of acute low salinity survival in *C. virginica* and results suggest that this trait is heritable and could be selectable in a breeding program.

Introduction

As a result of decades of overharvest, habitat destruction, and the introduction and spread of disease, wild Eastern oyster (*Crassostrea virginica)* populations have seen drastic declines across their native range (Beck et al., 2011). In order to support market demand, oyster aquaculture production has increased substantially since the 1970s (Green and Tracy, 2013). While success of the United States aquaculture industry has faced challenges from heightened environmental regulations, state laws, inadequate financing, and disease (Green and Tracy, 2013; Kennedy and Breisch, 1983), salinity is one of the most important environmental factors dictating successful cultivation and production of oysters in estuarine systems (Shumway, 1996). Low salinity negatively impacts Eastern oyster physiology by reducing clearance rate (Casas et al., 2018b; Loosanoff, 1952) and feeding rate (Casas et al., 2018b), which likely reduces growth (Loosanoff, 1952; Rybovich et al., 2016; summarized in Shumway, 1996) and overall productivity (Leonhardt et al., 2017).

Low salinity conditions in the mid to upper Chesapeake Bay, and in other coastal regions of the US, pose challenges for successful cultivation of oysters, but these regions may also provide a potential refuge from two major diseases affecting oysters, MSX (*Haplosporidium nelsoni*) and

Dermo (*Perkinsus marinus*). Dermo distribution and intensity are highly correlated with salinity, displaying lower intensities at salinities < 12 (Burreson and Ragone Calvo, 1996; Burreson et al., 1994; Bushek et al., 2012; La Peyre et al., 2003; Ragone and Burreson, 1993; Ray, 1954; Soniat, 1985). MSX is also a salinity-dependent disease and has lower intensities and subsequent oyster-related mortalities in lower salinity waters (Andrews, 1964; Farley, 1975; Haskin and Ford, 1982), and is completely non-existent at salinities below 10 (Andrews, 1983; Ford, 1985). While low salinity environments reduce the risk of mortality from disease, they also pose a challenge for production through physiological impairment.

An additional concern for oyster aquaculture in low salinity environments is freshet events that can cause extreme reductions in salinity over very short periods of time. In the upper Chesapeake Bay, where salinity is already at the lower end of the optimal range (optimal range 14-28, Shumway, 1996; Chester River to Crisfield 1 – 15, Maryland Department of Natural Resources), extreme low salinity (< 3) conditions typically occur in the spring following heavy rainfall and freshwater inflow from the Susquehanna River ("spring freshet", Andrews et al., 1959; Engle, 1946; Schubel and Pritchard, 1986; Southworth et al., 2017). Depending on the severity of the freshet, extreme low salinity conditions can last into the summer months (NOAA, 2019; Maryland Department of Natural Resources). The opening of the Bonnet Carré Spillway and diversion of freshwater in the spring of 1937, 1945 – 1947, and 1999 resulted in extreme low salinities and oyster mortality in the Mississippi Sound (Brammer et al., 2007; Butler, 1952b, 1949; Gunter, 1953), and estuaries in California and Oregon experience spring freshets following the distinct winter-wet season, which is a characteristic of Mediterranean climates (Cheng et al., 2015). Freshwater pulses have caused severe oyster mortalities in the Mid-Atlantic region (Andrews et al., 1959; Beaven, 1946; Munroe et al., 2013; Southworth et al., 2017), and low

salinities experienced during high-temperature, summer months can be especially detrimental, as temperature is particularly important in governing mortality at low salinity (La Peyre et al., 2013; Loosanoff, 1952).

Despite the critical role of salinity in governing oyster growth, disease, and in regulating reproduction and other physiological processes, relatively little research has focused on the underlying genetic architecture of salinity tolerance in the Eastern oyster. Previous studies examining natural populations of *C. virginica* have revealed that larvae have a narrow salinity tolerance and perform better in their natal environments compared to being cultured in other locations or at other salinities (Eierman and Hare, 2013; Newkirk et al., 1977; Scharping et al., 2019). Experimental work shows that larvae from low salinity populations within Delaware Bay have higher survival in low salinity conditions, while larvae from moderate to high salinity populations have similar survival across a salinity gradient, suggesting potential genetic differentiation of upstream, low salinity populations (Eierman and Hare, 2013). While multiple studies have hinted at the genetic component of low salinity tolerance and adaptation (Andrews et al., 1959; Leonhardt et al., 2017; Méthé et al., 2015; Newkirk et al., 1977; Southworth et al., 2017), few studies have actually examined the potential for breeding, or improving, low salinity tolerance in oysters, and no studies have investigated survival in extreme low salinity conditions (< 3).

To determine the heritability of acute low salinity survival in *C. virginica*, two low salinity exposure experiments were conducted at an extreme low salinity (2.5 – 2.7). Heritability of low salinity survival was estimated using mixed linear models on a binary trait, live or dead, using both a Bayesian and a restricted maximum likelihood approach. Hemolymph osmolality from individual oysters was simultaneously investigated to better understand osmoconformation in

real time during the experiment as a potential mechanism of differential salinity survival. This research is the first step in the future development of an oyster line equipped to handle acute freshwater pulses (< 3) for aquaculture in coastal systems, such as the upper Chesapeake Bay.

Methods

Production of low salinity lines and breeding designs

The diploid family breeding program at the Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science (VIMS) dates back to 2004 with the incorporation of the first founder populations. Founders, from both selected and wild populations, were introduced over multiple generations and hundreds of families are spawned and tested yearly in high and low salinity environments.

In mid-April 2016, ripe oysters from ABCs low salinity family population were strip spawned and crossed using a 2 X 2 design to create full-sibling larval cultures. In this design, every female is mated to two different males and every male is mated to two different females. Fertilized eggs were reared in 60L, aerated, larval culture barrels filled with filtered sea water. Larvae were fed daily a ration of microalgae, including *Pavlova* sp., *Chaetocerous neogracile* and *Tetraselmis* sp. Typically, the diet was 45/45/10% of each species, respectively, by day 12 and through metamorphosis to the eyed stage. The concentration of live algae fed started at 20,000 cells/mL and was increased to 65,000 cells/mL through the eyed stage and setting. Eyed larvae were removed from the culture and set in a downwelling system on ground oyster shell (micro cultch). When spat were large enough to be retained on a 500 μm screen, they were transferred to a land-based upwelling system where they fed on algae in raw water from the York River. Once spat had reached a shell height of 10 mm they were transferred to bags and then

relocated to the Coan River, an upstream tributary of the Potomac River in Maryland, USA. They were then transferred to Horn Point Laboratory, Maryland, USA in March 2017 where monthly salinity averaged 13.1 ± 0.14 standard error of the mean (SEM). Families were submerged in bags on rebar-racks in the Choptank River, and bags were flipped and cleaned monthly to remove biofouling and ensure adequate flow. Families were moved to an enclosed boat basin on Horn Point campus in November 2017 for overwintering. They remained in the boat basin until they were brought into the Horn Point Laboratory for experimentation in April 2018 at two years of age.

Acute low salinity experimental exposure

Adult oysters from the F1 families were 2-years old at the start of experimentation. For experiment 1, families had a mean height (umbo to ventral margin) of 85.27 mm (SD = 7.11), and family mean height ranged from 71.53 to 106.39 mm. For experiment 2, families had a mean height of 82.39 mm (SD = 8.66) and family mean height ranged from 61.29 to 103.56 mm. Sixty oysters per family were randomly divided among triplicate plastic baskets (20 each) secured to the bottom of custom-made Taylor floats submerged in 183-cm diameter tanks (~1800 L) located indoors at Horn Point Laboratory, Maryland, USA. Desired salinity was reached by mixing continuously flowing Choptank River water (salinity 7-11) and oxygenated, heated well water (salinity 0). The full experiment was performed twice at two seasonal time points, experiment 1 in April-May and experiment 2 in July-August, on different individuals from the same set of 50 families. Heated well water was used to maintain a temperature 26.3°C ± 0.067 SEM for experiment 1, and 27.0°C ± 0.089 SEM for experiment 2. Salinity, temperature, and dissolved oxygen were monitored continuously using Vernier LabQuest2 probes (Vernier Software & Technology, Beaverton, OR, USA) with measurements recorded every five minutes, and by

point sampling multiple times daily using a YSI-85 handheld multimeter (YSI Incorporated, Yellow Springs, OH, USA). Flow rates from raw ambient water and heated well water were adjusted as needed to maintain salinity within 0.5 of the target salinity.

Prior to experimental exposure, oysters were acclimated in floats under ambient salinity and temperature (10.0 ± 0.24 SEM and 9.26°C ± 0.61 SEM for experiment 1, and 8.4 ± 0.14 SEM and 26.3°C ± 0.28 SEM for experiment 2) for one week. Mortality during acclimation was < 1% for both experiments, and oysters that died over this period were replaced. Oysters that died over the acclimation period were usually full of mud or already dead when entering the Taylor float. Over a period of two days, salinity was gradually decreased by 4 salinity units/day to the desired level of 2.7 for experiment 1, and by 3 salinity units/day to the desired level 2.5 for experiment 2. Desired salinity was chosen based on previous mortality trends at low salinity (Southworth et al., 2017), and from pilot studies on wild Choptank River oysters and a random sample of the fifty half-sibling families showing close to 40% mortality at a salinity of 3 and temperature of 24.7°C ± 0.59 SEM over a two-week period. A salinity of 2.7 was chosen for experiment 1 with a goal of eliciting a mortality response in roughly half the population in an experimentally tractable timeframe (~2 weeks). Experiment 2 was informed by experiment 1 and salinity was reduced to 2.5 to elicit a stronger mortality response. Temperature was gradually increased over two days to achieve the desired level of 27°C for both experiments. A subset of 20 individuals from 7 of the experimental families were held in a similar custom-made Taylor float in a separate 183-cm diameter tank during each of the experimental exposures. The control tanks were held at ambient salinity (10.8 ± 0.06 SEM for experiment 1, 8.0 ± 0.05 SEM for experiment 2) and at a temperature of 26.9°C ± 0.14 SEM and 27.7°C ± 0.15 SEM for experiment 1 and 2,

respectively, to ensure experimental mortality was not related to elevated temperature, float structure, water input, or any additional variables introduced from the experimental set-up.

Exposure experiments lasted for thirty days to ensure mortality trends were adequately captured, and oysters in both the experimental and control tanks were monitored daily for mortality. Valve closure was noted but not quantitatively measured. Oysters that gaped (shells open) for more than five minutes after being removed from the water and perturbed by tapping the shell were collected and marked as dead (Lombardi, 2012). Day of death and shell height (mm), measured from the umbo to the ventral margin, for all dead oysters was recorded. At the end of the 30-day exposure, a subset of the remaining live oysters was measured to calculate mean height for each family. During the exposure, flow through Choptank River water supplied some natural phytoplankton, but diet was also supplemented each day with Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA) at a ration of 1.5% total dry tissue biomass per tank (Southworth et al., 2010; data from Thomas et al., 2019) and 2 L of live, cultured phytoplankton (*Tetraselmis* sp.) at a density of approximately 200,000 cells/mL, provided by the Horn Point Laboratory Oyster Hatchery. Flowing river and well water were shut off for a period of two hours during feeding. Tanks were drained and scrubbed weekly to remove biofilm and prevent the accumulation of sediment in the baskets and on the bottom of the tanks. Floats were rotated among tanks every three days during experiment 2 only after detecting a possible tank effect in experiment 1.

We used Wilcoxon rank sum tests to examine differences in environmental parameters between experiments (salinity, temperature (°C), dissolved oxygen (mg/L), and flow rate (s/L)). We examined differences in family survival (number dead) on three among-subject factors (family, tank, and replicate nested in tank) for experiment 1, and two among-subject factors

(family and replicate) for experiment 2 using two-way ANOVAs. All computations were performed using the R statistical software (version 3.6.1; R Core Team, 2019).

Narrow-sense heritability (h^2) estimation

Survival at low salinity is effectively a binary trait (live or dead) and is referred to as the liability or latent variable (de Villemereuil, 2012; Falconer and Mackay, 1996), which is a threshold trait with an underlying continuous normal distribution. Here, we assume the liability is multifactorial, resulting from both genetic and environmental components. We executed an animal model using a frequentist approach in ASReml-R (Butler et al., 2007; Wilson et al., 2010) and a Bayesian approach in MCMCglmm R package (Hadfield, 2010) to estimate narrow-sense heritability (h^2) for the liability, mortality at acute low salinity exposure, for each of the two low salinity exposure experiments. We used these packages to separate the observed variation in the phenotype into an additive genetic variance and a residual variance component (e.g. Kruuk, 2004):

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

where l_i is the liability or the phenotype of the individual, μ is the average population phenotype, a_i is the influence of the additive genetic effect of the alleles on the phenotype, and e_i is the residual effect accounting for the remaining variation, usually due to environmental factors. Residual variance was fixed at 1 because binary data does not provide enough information to infer liability variance (e.g. de Villemereuil et al., 2013).

Hierarchical models using binary data can be fit using either a logit link function for data with a categorical distribution, or a probit link function for data with an ordinal distribution

(Gelman and Hill, 2007). Both link functions, logit and probit, use a cumulative probability function to model a binary outcome and therefore result in very similar model outcomes and subsequent heritability estimations (Charmantier et al., 2011; Liao, 1994; Nakagawa and Schielzeth, 2010). Heritability estimates with either MCMCglmm or ASReml-R were nearly identical regardless of distribution chosen, so we only report the results from models with an ordinal distribution. Tank and replicate effects were initially incorporated to account for additional local environmental effects (Kruuk, 2004), but were ultimately excluded because they were not statistically significant.

For MCMCglmm, we used a X^2 distribution (V=1, nu=1000) with 1 degree of freedom for our prior because literature suggests that it handles binary data well (de Villemereuil, 2012). Fixing the residual variance to 1 for binary data leads to a non-symmetric calculation of heritability, and requires that the variance of the additive genetic component remains small (explained in Appendix B: de Villemereuil, 2012). We also estimated heritability with an Inverse-Gamma (V =1, nu = 0.002) prior to test for potential bias in the estimation of additive genetic variance due to an overly informative prior. The Inverse-Gamma prior has a very long tail and places most of its probabilistic weight on $h^2 = 1$, therefore making it overly informative. We ran the models for 5 million iterations with a thinning interval of 200 after a burn-in period of 1 million iterations to limit autocorrelation and obtain effective sample sizes > 2000. Convergence of MCMC sampling and autocorrelation of chains were assessed by examining the shape of the traces and posterior density following de Villemereuil (2012). We used model outputs to estimate narrow sense heritability (h^2), which is the proportion of the phenotypic variance (σ_P) that is attributed to the additive genetic variance (σ_A) (de Villemereuil, 2012; Wilson et al., 2010).

An additional source of variation is introduced from the link function (σ_{link}), which relates the linear model to the response variable and is used to fit the data (Breslow and Clayton, 1993), and thus needs to be accounted for in the heritability estimate (de Villemereuil, 2012). Heritability estimates from ASReml-R are measured on the observed scale, so we converted our observed estimates to the underlying liability scale, (equation 25.8b in Chapter 25 of Lynch and Walsh, 1998; Dempster and Lerner, 1950):

$$h^2 = [h_o^2 * (\Phi_p (1- \Phi_p))] / [p(x_p)]^2$$

Where the underlying heritability (h^2) is the product of the observed heritability (h_o^2) and the phenotypic variance ($\Phi_p (1- \Phi_p)$), where Φ_p is the proportion affected (percentage of dead individuals) in our population. That product is then divided by $[p(x_p)]^2$, where (x_p) is the estimated threshold from the mean liability, or the z-score which represents 1 – the proportion of our affected population (Φ_p), and $p(x_p) = (2\pi)^{-1/2} \exp(- x_p^2/2)$ (Lynch and Walsh, 1998; Van Vleck, 1972). We assessed the accuracy of heritability estimates produced by ASReml-R as previous literature suggests that Bayesian approaches produce more accurate heritability estimates for binary traits compared to Frequentist approaches (e.g. de Villemereuil et al., 2013). To do this, we produced 200 randomly simulated datasets with the same pedigree structure and heritability as in each of our ordinal experiments using MCMCglmm (0.34 for experiment 1, 0.59 for experiment 2). We ran each simulated dataset through both ASReml-R and MCMCglmm, and converted each ASReml-R heritability estimate to the underlying scale.

Genetic and phenotypic correlations

We used MCMCglmm and ASReml-R with an ordinal distribution to calculate the genetic covariance matrix between the two salinity exposure experiments to investigate the among-trial genetic correlation (r_g) for survival. For the MCMCglmm correlation model, we fit a dam and sire model to account for maternal effects. We used a slightly stronger prior (V = diag(2), nu = 2) and ran the model for 10 million iterations with a burnin period of 100,000 and a thinning interval of 200 to achieve minimal autocorrelation and an effective sample size > 10,000. We evaluated convergence of MCMC sampling and autocorrelation of chains following de Villemereuil (2012). The genetic correlation based on the additive genetic variation was calculated by (de Villemereuil, 2012; Falconer and Mackay, 1996):

$$r_g = \text{cov}_g / \sqrt{(\sigma_{g1} * \sigma_{g2})} + \sigma_{link},$$

where cov_g is the additive genetic covariance between the two experiments, σ_{g1} is the additive genetic variance of experiment 1, σ_{g2} is the additive genetic variance from experiment 2, and σ_{link} represents the appropriate variance introduced from the link function. Tank and replicate effects were excluded because they differed between experiments. A Spearman's rank order correlation test was performed using R Statistical Software on family cumulative survival data to investigate significant phenotypic correlations between the two experiments (version 3.6.1; R Core Team, 2019).

We executed bivariate (shell height and survival) animal models using MCMCglmm and ASReml-R for individuals with data for both traits for each experiment to test for genetic correlations between shell height and survival. Both methods were executed using the probit link

function, and MCMCglmm models were run for 6 million iterations with a thinning interval of 1000 after a burn-in period of 1 million iterations using a V=diag(2), nu=2 prior (de Villemereuil, 2012). We estimated genetic and phenotypic correlations between the two traits for each experiment and method using the random variance components.

Hemolymph osmolality

The change in oyster hemolymph osmolality was monitored during the salinity step-down (beginning of exposure experiment) and throughout the first eight days of exposure when salinity was static at ~2.5 for nine families during the second low salinity exposure experiment in July-August. The nine families were first chosen for sampling based on survival during the first experiment, and then selected for analysis based on survival during the second experiment. Samples were collected and processed on days -2 (i.e. 2 days prior to the start of the experiment), -1, 0, 2, 4, 6, and 8. For each sampling day, three individuals from each exposure replicate were sampled for a total of nine individuals per family. Oysters were sampled without replacement by notching the anterior axis and withdrawing 500 – 1000µL of hemolymph from the adductor muscle using a 27-guage needle according to McFarland et al. (2013). Paired hemolymph and water samples collected during each of the sampling days were frozen at -80°C for future analysis. Prior to notching, oysters were measured for height (mm, umbo to ventral margin). Hemolymph and corresponding water samples were analyzed to determine osmolality using a VaporPro® vapor pressure osmometer (Wescor Inc., Logan, Utah, USA) at the Institute for Marine and Environmental Technology in Baltimore, MD, USA. Mean water osmolality was subtracted from mean family hemolymph osmolality and one-way ANOVAs were conducted at each time point separately to examine differences in osmolality between families on each

sampling day. We also investigated the association of overall family survival rank (from 1 to 50, highest surviving to lowest surviving at the end of the experiment) and survival "category" (top, middle, and worst performing) with osmolality change for each given sampling day to determine if the osmoregulatory phenotype could be a predictor of family rank for survival during acute low salinity exposure using one-way ANOVAs.

Results

Survival during acute low salinity stress and comparison between experiments

The first experiment began in early April and temperature, salinity, and dissolved oxygen varied no more than 1% (Table 1). Major mortality began on day 6 of low salinity exposure, peaked at day 9 (82 oysters dead), and then tailed off around day 14 (Figure 1). The majority of mortality (78%; 535 oysters) occurred from day 6 to 18, and at least 10 oysters were recorded dead daily during this 13-day period (43% of experimental days, Figure 1). The second experiment began in mid-July and temperature, salinity, and dissolved oxygen remained consistent, similar to experiment 1, varying no more than 1% over the month-long exposure (Table 1). Mortality followed a similar trend to experiment 1, ramping up on day 6, peaking at day 10 and 11, and tailing off starting on day 14 (Figure 1). The mortality peak for experiment 2 was greater than experiment 1 and a maximum of 140 oysters were recorded dead on day 11 (5.6% of total remaining alive individuals). The majority of mortality (70%; 1110 oysters) occurred from day 6 to 18, and more than 10 oysters were recorded dead from day 3 to the end of the experiment on day 30 (93% of experimental days; Figure 1). Mortality in the control tanks was low throughout the experimental period, totaling 4% and 5% for experiment 1 and 2,

respectively, indicating that temperature alone was not a major source of mortality observed during the trials.

Table 1. Details of the environmental conditions for both experiments and results of statistical analysis to compare conditions between experiments. Start date, end date, mean salinity, mean temperature (°C), mean dissolved oxygen (mg/L), and mean flow rate (L/min) for each 30-day low-salinity exposure experiment ± SEM. Note that data exclude the 2-day salinity step-down.

			Average ± SE			
Experiment	Start Date	End Date	Salinity	Temperature (°C)	Dissolved Oxygen (mg/L)	Flow Rate (L/min)
1	4/5/18	5/7/18	2.7 ± 0.029	26.3 ± 0.0665	3.83 ± 0.0264	25.9 ± 0.775
2	7/17/18	8/18/18	2.5 ± 0.024	27.0 ± 0.0891	4.64 ± 0.0540	27.3 ± 0.841
			*	*	*	
			t(641.6) = 7.81, p = 2.28e-14	t(545.2) = -6.46, p = 2.23e-10	t(208.8) = -13.5, p = 2.20e-16	t(27.82) = -1.18, p = 0.247

* indicates significant difference in means between experiments.

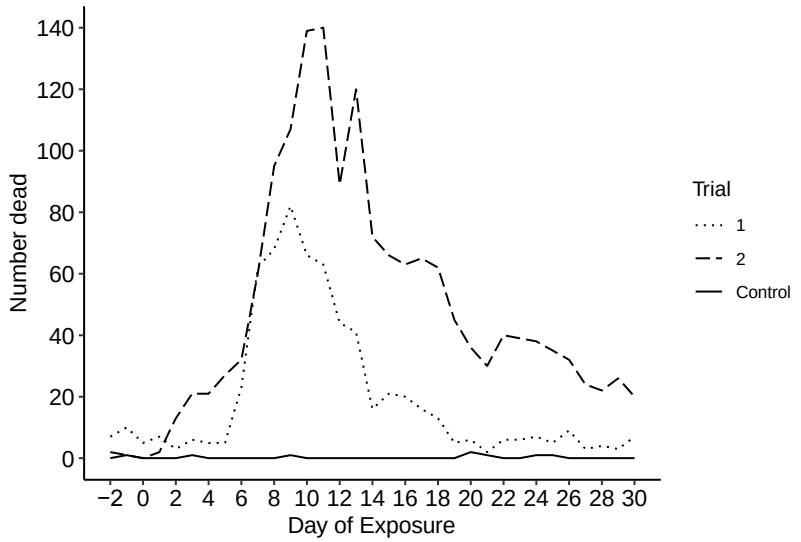

Figure 1. Total number of dead oysters from all families during the 30-day exposure to a salinity of 2.7 and 2.5 for experiment 1 and 2, respectively. Control oysters were kept at ambient salinity (10.8 ± 0.059 SEM) and 26.9°C ± 0.14 SEM during experiment 1 (control for experiment 2 was excluded due to similarity in trend for experiment 1, and for simplicity of the graph).

At the onset of each experimental exposure (day 0) when salinity reached 2.7 and 2.5, respectively, the majority of oysters had closed their valves. As the experiment progressed, oysters began filtering again and we observed new shell growth, suggesting that these oysters were able to exchange water and feed during exposure to extreme low salinity. For both experiments, there was a statistically significant effect of family on mortality (Figure 2A, experiment 1 ANOVA $F_{50,88} = 4.22$, $P < 0.00001$; Figure 2B, experiment 2 ANOVA $F_{49,100} = 5.67$, $P < 0.00001$), suggesting a strong underlying genetic component (family effect) for survival under low salinity conditions. Mortality among families in experiment 1 ranged from 0.05% to 57%, with a mean mortality of 23% (Figure 2A). In experiment 2, mortality ranged from 13.3% to 100%, with a mean mortality of 53% (Figure 2B). Overall mortality was significantly greater in experiment 2 (53%) than experiment 1 (23%) (ANOVA $F_{1,49} = 103.49$, $P<0.00001$) across all families (Figure 2C).

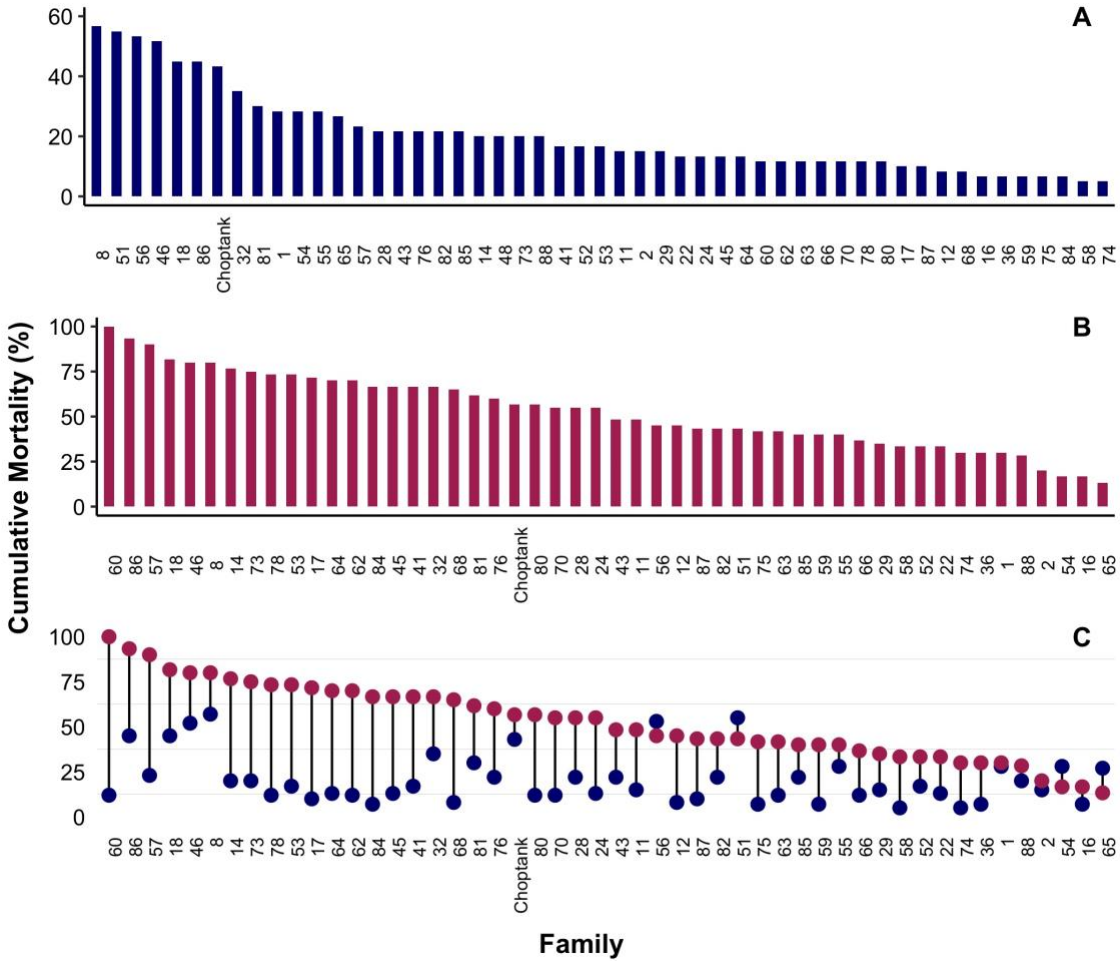

Figure 2. Plots showing cumulative mortality in the two low salinity exposure experiments. There was a statistically significant effect of family on mortality for A) experiment 1 (ANOVA; $F_{50,88} = 4.22$, $P<0.00001$), and B) experiment 2 (ANOVA; $F_{49,100} = 5.67$, $P<0.00001$). C) Lollipop plot showing significantly higher mortality for nearly all families in experiment 2 (maroon dots) compared to experiment 1 (navy blue dots).

Narrow-sense heritability (h^2) estimation

Estimates of narrow-sense heritability for survival in acute low salinity were significantly greater than zero for both experiments using both methods. For experiment 1, heritability estimate using MCMCglmm was 0.34 (95% credible interval (CI) 0.18 – 0.51), and underlying

heritability estimate using ASReml-R was 0.40 ± 0.05 SE (Table 2). Heritability estimates for experiment 2 were larger than experiment 1 using MCMCglmm (0.34 for experiment 1 and 0.59 for experiment 2), but were not statistically different from one another since the confidence intervals overlapped (0.18 – 0.51 for experiment 1, and 0.42 – 0.78 for experiment 2; Table 2). Similarly, heritability estimates for experiment 2 were nearly identical to the first experiment using ASReml-R (0.4), and were not statistically different from one another (SE 0.05 for experiment 1 and 0.15 for experiment 2; Table 2). The difference in point estimates from MCMCglmm and ASReml-R (underlying heritability) was larger for experiment 2 (38.4% and 16.2% different for experiment 2 and 1, respectively), but no conclusions can be drawn about the significance of this difference due to the fundamental differences in methods (e.g. 95% highest posterior density interval (HPDI) for MCMCglmm versus standard error for ASReml-R).

Table 2. Observed (h_o^2) and underlying (h^2) heritability estimates for experiment 1 and 2 using ASReml-R ± SE and MCMCglmm (95% confidence interval).

	ASReml-R		MCMCglmm
	Observed heritability (h_o^2)	Underlying heritability (h^2)	Estimated heritability (h^2)
Experiment 1	0.21 ± 0.05	0.4 ± 0.05	0.34 (0.18 – 0.51)
Experiment 2	0.26 ± 0.15	0.4 ± 0.15	0.59 (0.42 – 0.78)

Simulations of 200 datasets from MCMCglmm indicate that ASReml-R underestimates the heritability values for both experiments (Figure 3). For experiment 1, the median (underlying) heritability estimate from the simulations in ASReml-R (0.33) was slightly lower than the median heritability estimate from MCMCglmm (0.34), but both were either identical or nearly identical to the simulated value (0.34) (Figure 3). The median heritability estimate of the simulations was substantially lower from ASReml-R (0.40) compared to MCMCglmm (0.56) for

experiment 2, and the ASReml-R estimate was substantially lower than the simulated heritability value (0.59) (Figure 3).

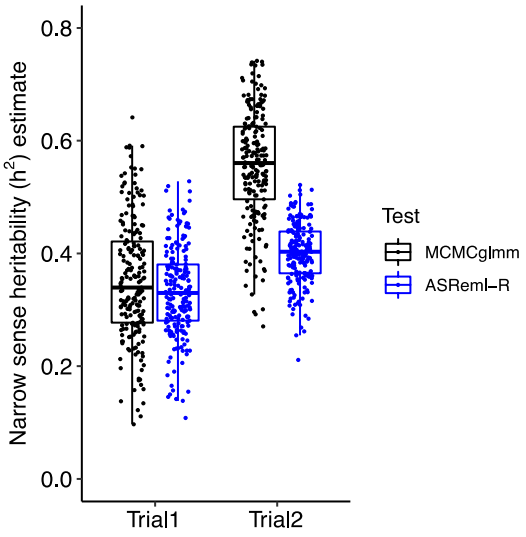

Figure 3. Boxplot depicting the median heritability estimates for 200 simulations of the MCMCglmm ordinal model output using both MCMCglmm and ASReml-R for experiment 1 and 2. Blue points represent underlying heritability estimates from each simulation in ASReml-R (median experiment 1 = .34, experiment 2 = .40), and black dots represent heritability estimates for each simulation in MCMCglmm (median experiment 1 = 0.35, experiment 2 = 0.56).

Genetic and phenotypic correlations

The genetic correlation between survival and shell height (mm, umbo to ventral margin) for individuals with data for both traits in experiment 1 was very low and not significant using both ASReml-R and MCMCglmm (ASReml-R -0.092 ± 0.19 SE, MCMCglmm 0.019 (-0.31 – 0.30)). For experiment 2, the genetic correlation between survival and height was moderate and significantly different than zero using both methods (ASReml-R 0.39 ± 0.15 SE, MCMCglmm 0.45 (0.073 – 0.74)). Genetic correlations for both experiments were very similar using both methods (0.07 and 0.06 difference for experiment 1 and 2, respectively). Similar to the genetic

correlations, phenotypic correlations between height and survival were low and insignificant for experiment 1 using both methods (ASReml-R 0.021 ± 0.034 SE, MCMCglmm 0.019 (-0.31 – 0.30)). On the other hand, phenotypic correlations for experiment 2 were low for both methods, and only significant using ASReml-R (ASReml-R 0.062 ± 0.037 SE, MCMCglmm 0.12 (-0.0010 – 0.23)). Phenotypic correlations for both experiments were very similar using both methods (0.002 and 0.058 difference for experiment 1 and 2, respectively).

Genetic correlations of survival between experiments were low and not significantly different from zero using both MCMCglmm and ASReml-R. For MCMCglmm, the genetic correlation for survival between experiment 1 and experiment 2 was -0.05 (CI -0.2 - 0.09) for the sire model and -0.014 (CI -0.17 – 0.13) for the dam model. For ASReml-R, the genetic correlation of the animal model for survival was -0.0151 ± 0.139 SE. Logliklihood did not converge for the ASReml-R model. The phenotypic correlation between experiments for mean family survival was not statistically significant (r_s = 0.21; p = 0.15).

Hemolymph osmolality

Measurements of oyster hemolymph osmolality at the beginning of the low salinity experiment (experiment 2) showed that the osmolality changed over time as water salinity decreased, but remained similar to the ambient water osmolality at all time points (ANOVA P > 0.05; Figure 4). At the start of the experiment (prior to step down), hemolymph osmolality was at its maximum level, averaging 268 mmol kg^{-1} across all families, and then decreased to an average of 94 mmol kg^{-1} across all families on day 0 when salinity reached 2.5 (Figure 4A). Hemolymph osmolality then remained relatively constant from days 0 to 8 while at a constant tank salinity of 2.5. While osmolality changed throughout the sampling period for all families, there were no significant differences in osmolality among families at any given timepoint.

Similarly, there was no significant association at any given timepoint between osmolality and rank of family survival or by survival category (top, middle, and worst performing). During the two-day step down, osmolality for nearly all families seemed to overshoot actual water osmolality (Figure 4B). After experimental salinity reached 2.5 on day 0, osmolality for all families was hyperosmotic to the water by no later than day 2 (Figure 4B).

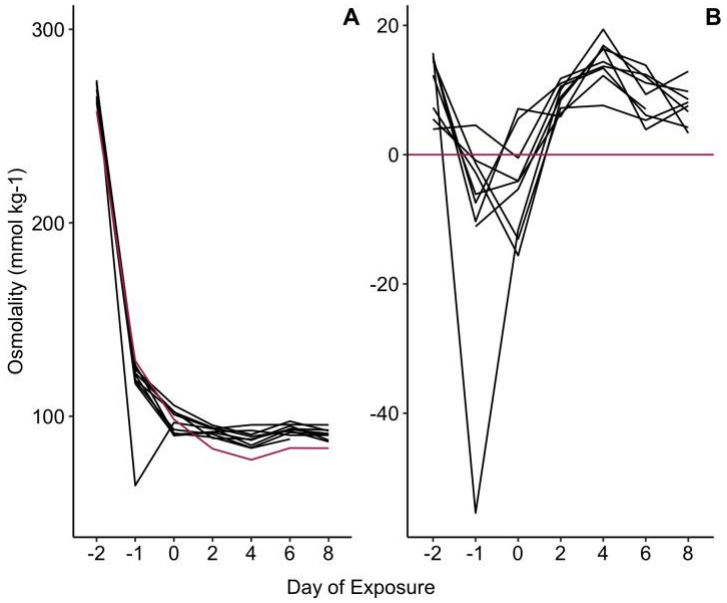

Figure 4. Mean hemolymph osmolality (mmol kg^{-1}) for nine families (depicted by solid black lines) collected A) during the 2-day salinity step-down, and on days 0, 2, 4, 6, and 8 of low salinity exposure during experiment 2. Mean water osmolality across all five tanks is presented by the red solid line. B) Mean water osmolality across all five tanks was subtracted from mean family osmolality at each sampling day, and therefore the red solid line represents the osmolality of the water.

Discussion

In response to targeted aquaculture in low salinity (low disease) areas prone to extreme freshwater pulses and to better understand the phenotype of survival in acute low salinity and the

potential for breeding, we performed two exposure challenges at acute low salinity. Results from both challenges show a strong physiological response to survival in acute low salinity conditions, with mortality one week after exposure. Significantly greater mortality was observed for experiment 2, which coincided with a period where oysters normally undergo gametogenesis, possibly contributing to additional stress. While analysis of hemolymph samples from experiment 2 suggests that oysters were able to rapidly conform to the low salinity conditions, hyperosmotic trends were observed for all families after salinity became static. Variation among families for cumulative mortality was observed in both experiments and narrow-sense heritability estimates for acute low salinity survival were 0.4 - 0.59, indicating strong potential for subsequent selective breeding of this trait.

Mortality trends and osmotic stress

Exposure of families to salinities of 2.5 – 2.7 produced significant, and consistent, mortality in each experiment, indicating that our exposure trials are highly suitable as a model for progeny testing. Mortality trends from both experiments followed a similar pattern, with the highest mortality occurring between days 8 and 12 of exposure. Similar mortality trends have been observed at day 7 for Eastern oyster seed (> 35 mm) in a low salinity (1-2) exposure experiment (Southworth et al., 2017), and a significant increase in mortality was observed for Olympia oysters after 8 days of exposure to a salinity of 5 (Cheng et al., 2015). Progeny testing for survival in acute low salinity conditions for adult oysters (~80 mm) need only last for a ~2-week timeframe, after which mortality is very minimal.

Osmotic stress may be a factor contributing to the observed mortality during our experiments, but the exact mechanism of death is unknown and may be multifactorial with a metabolic basis. *Crassostrea virginica*, like most marine bivalves, close their valves as a first

line of defense when exposed to stressful conditions (Shumway, 1996). Valve closure, even slightly, decreases water filtration and negatively affects feeding rates and gas exchange (Shumway, 1996; Casas et al., 2018). While we did not specifically quantify valve movement during either of our experiments, we observed open valves shortly after the onset of each experiment, suggesting that our mortality was not simply from prolonged valve closure. After initial valve closure, oysters can regulate their internal fluids to some degree by concentrating or diluting their extracellular fluid (hemolymph) by mobilizing intracellular free amino acids (FAA) to match the osmolality of the surrounding seawater as osmoconformers (Deaton et al., 1989; Deaton and Koehn, 1985; Pourmozaffar et al., 2019; Shumway, 1996), as shown by the similarity in hemolymph and water osmolality observed in our study.

At low salinities, osmoconformers take up water by osmosis and then decrease their cellular FAA concentrations by diffusion of solutes through excess water from swelling (Larsen et al., 2014; Lynch and Wood, 1966; reviewed in Pierce, 1982). This uptake of water creates a problem maintaining cell volumes efficient to sustain normal cell function and maintenance of cellular constituents (Shumway, 1996). Oysters may be able to handle the stress from the large amount of energy required for maintaining FAA pools, ion gradients, and for the uptake of water when osmoconforming to such low salinities for a short period of time (Shumway, 1996; Deaton et al., 1989), but this stress may become overwhelming and eventually detrimental if conditions persist. This threshold of osmotic stress and elevated temperatures may coincide with our observed peak in mortality during 8-14 days of both experimental exposures, where those individuals unable to handle the stress perish. Differences in salinity tolerance across species, populations, and individuals have thus been attributed to variation in dealing with intracellular FAA pools (Pierce, 1971a) through various bioenergetic approaches (Sokolova et al., 2012). The study of these

mechanisms and FAA pools will be important future work for understanding salinity tolerances and osmoconformation in light of the review published by Pourmozaffar et al. (2019).

Genetic and phenotypic correlations

Additional stress stemming from gametogenesis likely caused higher cumulative mortality in experiment 2 versus experiment 1 (23% vs 53%, respectively). We saw negligible mortality in control tanks for both experiments, indicating that temperature was not the sole factor driving our observed mortality. During daily mortality monitoring for experiment 2, we noted that many individuals had full, 'ripe' gonads and others were translucent from recently spawning. Experimentally, mortality has been observed when moving spawning oysters to a salinity of 3 (Loosanoff, 1952). Summer mortality events have been extensively documented in cultured blue mussels in the Magdalen Islands and off the Coast of British Columbia in late July resulting from high temperatures coinciding with major spawning events (Emmett et al., 1987; Myrand et al., 2000; Myrand and Guadreault, 1995; Tremblay et al., 1998). "Summer mortality" syndrome in the Pacific oyster (*Crassostrea gigas*) has been documented off the coast of France since 2008 and has been attributed to elevated temperature (> 19°C) and physiological stress associated with gonad maturation (Samain and McCombie, 2008), as well as increased susceptibility to infection from pathogens resulting from high temperatures (17-24°C) (Pernet et al., 2012; Petton et al., 2013). Another study showed that *C. gigas* displayed a reduction in reserves for protein synthesis, lowered immunocompetence, and lowest glycogen levels post-spawning (Li et al., 2007), indicating a decrease in resiliency for future disease infection. Gametogenesis and spawning are energetically costly processes that deplete bivalves of their reserves leaving them more susceptible to additional environmental stressors (Lambert et al., 2008; Li et al., 2007; Myrand et al., 2000). Therefore, the timing of low salinity events and the

physiological state of the oyster are critical to understanding osmotic tolerance in oyster populations.

Low estimates of phenotypic correlations between shell height and survival for both experiments suggest that these two traits are poorly correlated at acute low salinity. The phenotypic correlations for both experiments were very weak (<0.12 for both methods and experiments), but were slightly significant for experiment 2 using ASReml-R (0.062 ± 0.0369). This slightly significant correlation may reflect the overall higher mortality observed during this experiment (more discrimination among families), or could reflect the larger metabolic and energetic demands required by larger oysters (Kooijman, 2010). Previous studies have found that market-sized oysters were more sensitive to low salinity and high temperatures than seed (La Peyre et al., 2013; Lowe et al., 2017; Rybovich et al., 2016). Larger oysters have less gill surface area per body weight available for gas exchange (Shumway, 1983), making it more difficult for them to meet tissue oxygen demands as temperature increases (Pörtner, 2010, 2002; Rybovich et al., 2016; Shumway, 1983). Clearly, mortality from acute low salinity exposure is multifactorial and larger individuals may be more susceptible to mortality due to their high energetic demands, especially when undergoing gametogenesis and spawning.

While our phenotypic and genetic correlations were quite similar for experiment 1 (all < 0.1), in experiment 2, genetic correlations were moderate (0.39 for ASReml-R and 0.45 for MCMCglmm) though phenotypic correlations were still very low (0.062 for ASReml-R and 0.12 for MCMCglmm). The average absolute disparity ($D_{abs} = |r_p - r_g|$, Willis et al., 1991) between genetic and phenotypic correlations for experiment 2 was high, ~ 0.3, and could be a result of sampling error of the r_g (Kruuk et al., 2008). Additionally, a large sample size per group (i.e. per number of parents or from each family) is required in order to estimate genetic correlations with

accuracy and our experimental sample sizes per family were quite small, ranging from 56-60 and 45-71 individuals for experiment 1 and 2, respectively (Robertson, 1959). The relationship between phenotypic and genetic correlations most often holds true for traits with high heritability (i.e. morphological traits, Hadfield et al., 2007), and our heritability estimates for shell height were quite high for both experiments (0.79 and 0.97 for ASRemlR and MCMC Trial 1; 0.55 and 0.67 for ASReml and MCMC Trial 2), while our heritability estimates for survival were moderate (0.4 – 0.59). Additionally, the discrepancy between correlation estimates could be attributed to a relationship between the environmental and genetic effects, which may work in opposing directions, therefore resulting in a small phenotypic correlation (Hadfield et al., 2007). Small sample sizes, sampling error of the genetic correlation, high heritability estimates for shell height, and potential interactions between environmental (i.e. gametogenesis) and genetic effects could all be responsible for the discrepancies between our phenotypic and genetic correlations for experiment 2, and therefore the estimates produced should be taken with caution. While phenotypic correlations have long been accepted as a proxy for genotypic correlations ('phenotypic gambit', Cheverud, 1988; Grafen, 1984; Sodini et al., 2018), further research is needed with appropriate sample sizes to assess both the genetic and phenotypic correlations between these two traits in acute low salinity, as size and survival are both important factors in aquaculture.

In general, one could expect family survival across the two experiments to be correlated since individuals from the same families were exposed to the same conditions, but experiment 1 may have more effectively captured the effect of survival to acute low salinity, while experiment 2 captured a more multifactorial stressor, survival in acute low salinity while undergoing gametogenesis. The difference between environmental factors, including those not directly

sampled, and physiological state of the oyster are reflected in our insignificant genetic correlations and weak family mean correlations in family survival between the two experimental trials. Therefore, it might not be appropriate to specifically test for survival at acute low salinity when gametogenesis becomes a complicating factor in the summer, as the model for the progeny test will be less useful. However, the timing of low salinity events should be taken into consideration for each breeding program on a site-specific basis, as late-summer or early-fall freshets may be more common in other locations. In Maryland, large drops in salinity are more likely in the spring when heavy rains are common (Schubel and Pritchard, 1986), so April might be a more relevant time for conducting these trials for a Maryland-specific breeding program.

Hemolymph osmolality and osmoconformation

The osmolality of the hemolymph samples collected during the salinity step-down and throughout the first eight days of exposure indicate that oysters were open and conforming to the changing salinity of the water (Figure 6), which is possible considering oysters can feed at salinities as low as 1 (Southworth et al., 2017). In this study, we observed open valves and new shell growth at the end of each experimental period at salinities of 2.5-2.7. The gradual step-down may have assisted with the conformation of hemolymph osmolality to water osmolality, especially at such a low salinity. Hand & Stickle (1977) showed that oyster pericardial fluid tracked that of water salinity as it was gradually changed from 20 to 10 and back to 20 over 24 hours, but an abrupt change in salinity from 20 to 10 caused valves to close for 19 hours. While differences in the duration of valve closure among families were not quantified, hemolymph osmolality suggests that valve closure did not inhibit adjustment to the experimental salinity.

Once salinity reached 2.5, the hemolymph of all families was slightly hyperosmotic (Figure 6B). Hand and Sickle (1977) saw a similar hyperosmotic trend in pericardial fluid during the

salinity step-down, and McFarland and colleagues (2013) saw hyperosmotic hemolymph for 2 and 6 days after an exposure to a salinity of 10 and 5, respectively. Slightly hyperosmotic trends are typically seen in osmoconformers (Pierce, 1982) and may suggest a delay in ion regulation during drops in salinity and at extreme salinities, especially during short exposures. Measuring differences in FAA pools or the relative concentrations of specific ions (e.g. potassium) may provide a more refined explanation for our observed differences in phenotypes across families, and is future research worth pursuing. Our results expand on the body of literature showing that oysters are able to conform to changing water salinities if shell valves are open and if the change is gradual (Hand and Stickle, 1977; Shumway, 1977a, 1977b), even at salinities below typical tolerance ranges.

Narrow-sense heritability (h^2) estimation of low salinity survival

Estimation of narrow-sense heritability using two distinct approaches produced highly significant values, indicating a strong genetic basis for survival in acute low salinity in the Eastern oyster. Survival in acute low salinity is a good candidate for a selective breeding program. For ASReml-R, underlying heritability estimates were the same for both experiments (0.4), while observed heritability estimates differed only slightly (experiment 1 $h_o^2 = 0.21$, experiment 2 $h_o^2 = 0.26$, Table 2). The lack of difference between underlying heritability estimates across experiments may be due to the almost identical observed estimates, but is more likely due to the bias and inconsistencies introduced when converting between observed and underlying heritability (Lynch and Walsh, 1998). The relationship and conversion between observed and underlying heritability depends on the sample incidence (Φ_p) in the population in a nonlinear fashion, since the probability of expressing a liability is bounded between 0 and 100% (Lynch and Walsh, 1998). Large overestimation of underlying heritability from observed

heritability has been noted in previous studies using parent-offspring regression when the proportion of the population affected was small, especially when Φ_p was smaller than 0.25 and larger than 0.75, and when the underlying heritability was large (Van Vleck, 1972). While we executed an animal model in our study, our Φ_p in experiment 1 (23%) may have resulted in the overestimation of the underlying h^2 (0.4) from the observed ($h_o^2 = 0.21$) if we consider the estimated h^2 from MCMCglmm to be the correct heritability (0.34, Table 2). On the contrary, the underlying heritability for experiment 2 was lower than the estimated (ASReml-R $h^2 = 0.4$, MCMCglmm $h^2 = 0.59$, Table 2), which may be due to the inherent difficulties associated with using ASReml-R to fit models with binary data.

As noted in the Results Section 3.2, there was a rather sizeable difference in estimates between the two methods, ASReml-R and MCMCglmm, for experiment 2, but the difference decreased when converting from the observed (h_o^2) to the underlying scale (h^2) for ASReml-R (Table 2, Experiment 2 observed difference = .33, underlying difference = 0.19). While we converted our observed heritability to the underlying scale (as reviewed by Lynch and Walsh 1998), this conversion is poorly documented for threshold traits in the peer-reviewed literature, especially when estimating heritability for non-disease traits in organisms other than humans. Therefore, it is important to briefly discuss this in some detail and highlight the pros and cons of these methods when estimating heritability of threshold traits in aquaculture species.

The discrepancy between heritability estimates from the two methods is explained by the difference in methods used by ASReml-R to estimate model parameters from binary data, 0 or 1. ASReml-R uses an approximate likelihood technique called penalized quasi-likelihood (PQL), also known as Schall's technique (Schall, 1991), which requires specific integration techniques for binary data in order to calculate the loglikelihood and produce model outputs (Breslow and

Clayton, 1993). Alternatively, Bayesian procedures avoid the need for numerical integration because they repeatedly sample the posterior distribution according to a pre-specified prior and the observed data (Breslow and Clayton, 1993). The PQL method utilized in ASReml-R underestimates the variance components for binary data (Breslow and Clayton, 1993; Rodríquez and Goldman, 2001), resulting in an underestimation in the observed narrow-sense heritability estimate (Charmantier et al., 2011). Recently, de Villemereuil and his colleagues (2013) elegantly showed the underestimation of observed heritability estimates (h_o^2) when using PQL on data simulated from known heritability values using a series of simulation studies. Additionally, they found that the degree of underestimation in ASReml-R increases as the true heritability value increases. The inherent underestimation of the random effect variance components from ASReml-R likely explain the large difference in our observed heritability estimations for both experiments (experiment 1 difference = 0.13, experiment 2 difference = 0.33), and the larger degree of underestimation for experiment 2 was likely due to the larger heritability (MCMCglmm $h^2 = 0.59$). Our simulation studies show that ASReml-R indeed underestimated heritability compared to MCMCglmm for the same datasets for both experiments, and that the degree of underestimation increased as the true heritability increased (Figure 3). Estimating heritability for binary traits is more complicated than for quantitative traits and currently appears to be inconsistently addressed in the literature. Results from this study support previous findings suggesting that observed heritability estimates from ASReml-R may be biased due to model-fitting methods in some cases, and additional sources of bias are introduced when converting between observed and underlying heritability. Therefore, MCMC-based animal model approaches may be more accurate when estimating heritability for a binary trait.

Prior selection is very important for Bayesian models and we briefly discuss the potential effects of different prior selection on our heritability estimates from MCMCglmm. Typically, flat non-informative priors are desired in order to induce little prior knowledge when estimating posterior parameters, therefore resulting in posterior estimates dominated by the observed data (Clark, 2005; McCarthy and Masters, 2005). Heritability estimates using both a X_1^2 distribution (V =1, nu =1000) and an Inverse-Gamma (V =1, nu =0.002) prior were very similar for both experiments, which is expected as the influence of the prior distribution decreases as sample size increases (de Villemereuil et al., 2013). But, for both experiments, models ran with an Inverse-Gamma prior had very small effective sizes (< 400) and the trace of the additive variance contained flat portions where the chain was stagnant for periods of time. Additionally, the density plot for experiment 2 was largely biased towards 1. De Villemereuil and colleagues (2013) investigated the use of different priors when estimating heritability from binary data and determined that the X^2 distribution with 1 degree of freedom (V =1, nu =1000) had the closest overall distribution to a uniform distribution on heritability (Figure 2 from Appendix B, 2013). Our small effective sizes (< 400) and flat portions in the trace of the additive variance using the Inverse-Gamma prior is indicative of a strong autocorrelation between runs. Therefore, the Inverse-Gamma prior has an influence on the estimated posterior distribution. These results provide further evidence that the X^2 distribution is preferable for binary data.

Implications for breeding of acute low salinity survival in oysters

The results of our acute low salinity experimental exposures provide important initial insight into the phenotype of acute low salinity survival and its underlying genetic architecture, yet it remains an open question whether or not this trait (survival at acute low salinity < 3) is distinct from less extreme, low salinity tolerance (e.g. 5-10). Genomic studies on the Olympia

oyster (*Ostrea lurida)* revealed differences in magnitude of gene expression among distinct, low salinity-tolerant populations in northern California following a freshet event (Maynard et al., 2018), and Butler (1949) suggested a minimum salinity of 6 for low salinity tolerant populations of eastern oysters in Louisiana. There is a clear underlying genetic basis for survival at high versus low salinity, which was the motivation behind the creation of both a low (8 – 15) and high (18 – 23) salinity breeding program by the ABC program at VIMS.

The results from our acute low salinity exposure experiments are applicable for populations of oysters that live in areas where freshwater inputs from local rainfall, storm events, and water management infrastructure leave oysters exposed to salinities < 3 for short (1 – 4 week) periods of time, such as those in the northern, Maryland portion of the Chesapeake Bay, and in other estuarine and coastal systems (i.e. Louisiana, FL, NY, CA). Extreme low salinity conditions for extended periods of time (e.g. days to weeks) will most likely have negative effects on feeding, growth, overall production and meat yield, making these regions more challenging for consistent oyster aquaculture production. It is also important to think about seasonal timing of these freshet events, as a freshet occurring in the winter or spring could affect the timing and progression of reproduction, whereas a freshet event in the summer could result in more substantial mortality events. Previous work suggests a benefit of winter and spring freshet events for reducing *P. marinus* infection intensity, prevalence, and subsequent oyster mortality (La Peyre et al., 2003), and recent work in Breton Sound, LA indicate fewer negative impacts on oyster growth and survival when extended low salinity events occur in the late spring and early summer when water temperature is < 25°C (La Peyre et al., 2013). Oysters selected for these extreme low salinity conditions could be interbred with other low or moderate salinity-tolerant populations to increase their adaptability and fitness in the event of a freshwater event. Understanding the mortality

trends, shifts in osmolality, and other physiological traits at extreme salinities could provide a more detailed understanding of salinity tolerance in general and the potential limits to adaptation and stress tolerance in this highly adaptive species. Furthermore, salinity tolerance might be a product of multiple physiological or genetic pathways and understanding which are most important and how they respond to salinity stress could enhance future breeding programs.

Genomic analysis of the genes or gene regions associated with acute salinity survival or susceptibility would be a logical next step to further our understanding of this trait. Results from our exposure experiments reveal that oysters can survive in nearly freshwater conditions for up to a month, which is longer than most naturally occurring freshwater events in the wild, though we have recently seen an unusually long period of low salinity in the upper Chesapeake Bay in 2019 (NOAA, 2019). Due to these changing conditions, our results are timely and it is worth considering the role of plasticity when it comes to salinity tolerance in oysters and how to breed for variable salinity stress (high or low) as our climate continues to become more variable with more severe weather events. It is especially important to think about salinity tolerance for populations in Maryland, as annual mean precipitation and temperature have been above average this century, and are predicted to continue increasing (Runkle et al., 2017).

Conclusions

The results from this study suggest that survival in acute low salinity is a heritable trait and suitable for selective breeding. Results also suggest that progeny testing for a 2-week timeframe at our experimental temperature (27°C) and salinity (2.5) will efficiently capture mortality trends for adult individuals (~80 mm in shell height). The differences in estimates between our experiments suggest that a program selecting exclusively for acute low salinity survival should be conducted when additional, potentially confounding, effects are minimal (i.e. temperature,

gametogenesis, etc.), and progeny testing should be conducted on a site-specific basis depending on the timing of local freshet events. When estimating heritability of binary traits, such as acute low salinity survival, it is important to consider the potential pitfalls of the methods available and to run multiple analyses with varying approaches to ensure the estimate is accurate. As aquaculture expands into lower salinity regimes, there is a greater need to understand the metabolic and physiological mechanisms (regulation of free amino acids pools) underlying optimal survival and growth in these regions.

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Chapter 3: Evaluating a progeny test and investigating physiology associated with survival in extreme low salinity for the eastern oyster *Crassostrea virginica*

Abstract

Low salinity negatively impacts 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 increase, coastal aquaculture operations face increased risk of prolonged exposure to extreme low salinity conditions. While recent research has determined that survival in extreme low salinity (< 3) is a moderately heritable trait in the eastern oyster, further challenges were conducted to investigate the importance of challenge duration and temperature on the survival phenotype and estimation of its genetic parameters. Growth and feeding rate were also assessed to investigate physiological phenotypes associated with differential survival in extreme low salinity. Individuals from 51 half-sibling families were exposed to either a short-term low salinity (2.5) challenge at constant temperature (27°C) for 2 months, or a chronic 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 (2 month) and chronic (6 month) low salinity challenge, and narrow-sense heritability estimates were similar for both challenges (short-term $h^2 = 0.35$, chronic $h^2 = 0.4$). A strong phenotypic ($r_S = 0.89$) and genetic ($r_G = 0.81$) correlation for family mortality were found between challenges. Non-linear curves fit to the rate of algal removal by individual oysters during a 24-hour feeding experiment revealed differences in feeding among families that were correlated to, and predictive of, family survival in the chronic low salinity exposure. Growth was negligible during the chronic challenge. Overall, 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 feeding rate and the parameters derived from the feeding curves may be used as a phenotypic proxy for longer-term survival during the extreme low salinity challenge.

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, 1952a, 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 (*Crassostrea virginica*) 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 (e.g. 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; reviewed in Du et al., 2021; Gledhill et al., 2020; Munroe et al., 2013; Southworth et al., 2017).

Understanding the physiological changes of oysters in response to extreme low salinity may shed light on the direct mechanisms responsible for survival in low salinity. In eastern oysters, valve opening, respiration, feeding, and clearance rates decrease with decreasing salinities (Casas et al., 2018b; Loosanoff, 1952; Shumway and Koehn, 1982), and one study suggests that feeding ceases completely at salinity <3 (Loosanoff, 1952). A recent modeling study predicted that low salinity impacts physiology and life history traits mainly through effects

on filtration rates (Lavaud et al., 2017). Decreased valve opening and the inability to filter, or the inability to filter effectively, in extreme low salinities would result in a deficit of energy required to maintain necessary biological functions, which may inevitably result in mortality. Therefore, measuring the removal of algae from the water over time is a phenotype worthy of examination in the context of low salinity testing and breeding. Additionally, a filtration/feeding phenotype may serve as a proxy for overall low salinity performance or survival, making for a more rapid and cost-effective test of individuals at low salinity.

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). While avoiding coastal environments that frequently experience extreme low salinities might seem like an obvious solution to a farmer, coastal regions are considered more suitable for oyster aquaculture due to the consistency of dissolved oxygen levels, as offshore (deep water) is associated with lower dissolved oxygen in the summer (Beckensteiner et al., 2020; Theuerkauf and Lipcius, 2016). Moreover, coastal areas with lower salinity conditions, salinity ~ < 12, can provide a refuge from some predators and the parasites *Haplosporidium nelsoni* and *Perkinsus marinus*, which cause MSX and Dermo disease, respectively, in oysters (e.g. Andrews, 1964; Burreson and Ragone Calvo, 1996; Shumway, 1996). Dermo outbreaks can result in greater than 50% mortality for a localized population or aquaculture operation (Craig et al., 1989). Thus, these low salinity regions provide a refuge from disease and increase the chances of individual oyster survival.

Survival in extreme low salinity has a strong genetic component. Low salinity survival was determined to be a different trait than high salinity survival (Allen et al., 2021). Additionally, a series of extreme low salinity challenge experiments (< 3) revealed differential survival among half-sibling families selectively bred for low salinity survival (McCarty et al., 2020) and between four F_2 families (McCarty et al., 2021). Results also suggest that survival in extreme low salinity is a moderately heritable trait ($h^2 = 0.4 – 0.5$), and adult oysters (> 80 mm) can survive in these extreme conditions for a full week before significant mortality starts to occur (McCarty et al., 2021, 2020). A moderate heritability value for a trait implies that differences in the genetics underlying that trait account for the observable differences in the trait phenotypes (Falconer and Mackay, 1996). Thus, survival in extreme low salinity is a good candidate for incorporation into a selective breeding program.

Selective breeding programs can be labor intensive and costly to run due to the challenges associated with phenotyping animals and developing reproducible progeny tests that yield high-quality phenotypic data. Initially, an appropriate and effective progeny test has to be carried out in order to elicit a variable phenotypic response across related individuals to make estimates of the genetic parameters underlying a trait (Falconer and Mackay, 1996; Lynch and Walsh, 1998). These tests require adequate facilities or grow-out sites to hold a large number of testing individuals that have a known (pedigree) or determinable (molecular marker-based) relationship structure (Allen et al., 2020). Field or laboratory-based trials can be costly due to the large sample sizes and large genotyping effort required to achieve enough power, as well as the personnel required to monitor and maintain animals during the trial. Moreover, traditional pedigree-based tracking requires more space and effort because families must be kept separate at all times (Allen et al., 2020). Trials performed in a laboratory setting may incur additional costs,

such as access to a natural or supplemented food supply, supply and filtration of natural seawater, and other necessary experimental adjustments (i.e. oxygen, temperature, salinity). A progeny test for extreme low salinity was previously developed (McCarty et al., 2020), but we are also interested in how a longer challenge with naturally fluctuating (ambient) temperature might be more realistic given the acuteness (1 month) and relatively high temperature of previous challenges. Performing a longer challenge at fluctuating temperatures permits the examination of extreme low salinity exposure duration and exposure temperature on survival, as the impact of temperature is a critical factor that drives mortality during low salinity exposure events (La Peyre et al., 2013; Loosanoff, 1952; Southworth et al., 2017).

In order to fine-tune a progeny test for survival in extreme low salinity and to investigate additional phenotypes related to low salinity performance, we performed two challenges (salinity < 3) for 2 and 6 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, feeding rate and growth were recorded for a subset of individuals. In addition to fine-tuning the low salinity challenge conditions, this work attempts to understand how key physiological phenotypes of oysters (i.e. removal of algae from the water column) may be associated with performance under low salinity stress.

Methods

Production of low salinity lines and breeding design

Full-sibling diploid families were created at the Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science in mid-April 2018 (Allen

et al., 2021). In brief, animals from the ABC low salinity family lines were strip spawned and mated in a 2 x 2 design, where every female is crossed with 2 different males and every male is crossed with 2 different females. Therefore, some families may share either parent with another family, making them half-siblings. Larvae were reared and fed following 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), MD, USA in March 2019. Families were brought immediately into the laboratory and kept in tanks with flow-through, ambient water from the Choptank River, MD, USA (salinity 9.76 ± 0.11 standard error of the mean (SEM), temperature 6.56 ± 0.28 °C SEM) until experimentation began in April 2019.

Laboratory-based low salinity challenges

Two low salinity challenges were conducted following the same procedures described by McCarty and colleagues (2020), but with a few modifications. Before the challenge began, oysters were approximately 1-year old and averaged 38.10 ± 0.18 mm SEM. The first challenge (referred to as the 'chronic' challenge) began on April 1st, 2019 after oysters from the 51 families were acclimated in Taylor floats for a week under laboratory conditions. For each family, oysters were separated into two identical-sized replicates depending on the total number of individuals, which ranged from 110–210 animals total. After 29 days in the chronic challenge, 60 oysters from each family were removed for the short-term challenge (see below for details). Replicates were randomly assigned to plastic baskets within one of twelve floats for each family, 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 2.51 ± 0.03 SEM. 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 6.78 ± 0.11 SEM) 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, Chesapeake Bay. Over the chronic challenge period, temperature gradually rose and fluctuated from 7.9°C to 30.3°C, with temperature peaking on July 21st. Oysters remained at this desired salinity and corresponding ambient temperature for 168 days (~ 6 months). Across all tanks, water flow averaged 0.476 L s^{-1} ± 0.00928 SEM and dissolved oxygen averaged 6.58 mg L^{-1} ± 0.054 SEM throughout the challenge. Oysters were inspected weekly to check for mortality and shell height (hinge to bill) was recorded for all dead individuals. During the challenge, flow-through Choptank River water supplied some natural phytoplankton, but diet was also supplemented every 3 days with Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA) at a ration of 1.5% grams of oyster dry weight. Flowing river and well water were shut off for a period of two hours during 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 (mm) to track growth throughout the duration of the experiment.

A 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), were maintained in a separate float and tank with continuous, flow-through river water at ambient salinity and temperature ranging from 5 – 11.5 and 7.9°C – 30.3°C, respectively, for the duration of the chronic exposure. Dissolved oxygen averaged 7.18 mg L^{-1} ± 0.13 SEM in the control tank during

the 6-month exposure period. Mortality was not directly measured for these individuals considering the very minimal mortality observed in two previous experiments (McCarty et al., 2020), but monthly assessment of these individuals during growth measurements revealed very minimal, if any, mortality. Growth was assessed monthly during the 6-month exposure on a total of 150 individuals, 25 from each of the half-sibling families.

On April 29, 2019, 30 oysters from each replicate, totaling 60 per family, were removed from the chronic low salinity challenge and transferred for a separate, short-term low salinity challenge (referred to as the 'short-term' challenge). Individuals were removed from the chronic challenge at a salinity of 2.7 ± 0.10 SEM and temperature of 17.9°C ± 0.03 SEM on transfer day (day 26 of chronic exposure). Mortality before being transferred to the short-term challenge was very minimal, 1.82% of the total starting population (mortality was low during this period because ambient temperature was <20°C; e.g. Southworth et al., 2017). For the short-term challenge, nine plastic baskets in three Taylor floats were divided into two halves using 1-inch vinyl coated wire mesh cage material. Families (no replicates) were randomly assigned to a plastic basket section and all three floats were placed in the same 6-ft diameter tank already at the target salinity (~ 2.5) and temperature (~ 27 °C). Salinity and temperature were maintained at 2.41 ± 0.30 SEM and 27.8°C ± 0.19 SEM, respectively, throughout the short-term challenge. Salinity and temperature levels were assessed daily with a YSI-85 and adjusted to maintain within 1 ppt and 1°C. Dissolved oxygen was monitored daily and averaged 6.41 mg L^{-1} ± 0.063 SEM throughout the challenge. Floats were pulled and mortality was checked every 4 days for 60 days. Feeding was identical to the chronic challenge, except supplementation with Shellfish Diet 1800® occurred every other day to replicate the feeding schedule during the two challenges

from McCarty et al. (2020). Water flowed through the tank at an average of 0.527 L s^{-1} ± 0.0108 SEM throughout the short-term challenge.

Statistical analyses for low salinity challenges

A two-way ANOVA was conducted to analyze the effect of family (51 families) and sampling month (7 time points) on individual height (mm; 50 individuals per family) during the chronic low salinity challenge to investigate differences in growth across families. A separate two-way ANOVA 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 chronic challenge were assessed using a Spearman's rank correlation test in the 'Hmisc' package ('Hmisc' version 4.3; Harrell, 2021). All computations were performed using the R statistical software (version 3.6.1; R Core Team, 2020).

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). 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 ('Hmisc' 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; R Core Team, 2020).

Feeding rate experiment

Removal of algae from the water column (feeding) was measured for individual oysters at four time points during the chronic exposure. Oysters were exposed to the chronic challenge for five weeks before feeding (algal removal) was assessed. Three to five individuals per family for 31 of the 51 families were examined for feeding behavior 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 chronic 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 chronic 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 chronic challenge (35 – 63% cumulative survival) and the remaining 16 families had cumulative survival values between the top 10 and bottom 10 performing families. Artificial seawater for the experimental beakers was produced by salting deionized water to a salinity of 2.5 with Crystal Sea® Marinemix (Marine Enterprises, Baltimore, MD), and a bubbler was added to each beaker to supply air (oxygen) and keep phytoplankton mixed. Water temperatures ranged from 20.7°C to 24.7°C across the four experimental days. A subset of individuals was tested on multiple sampling days to investigate how day and temperature affected individual feeding performance.

On each experimental day, live algae (*Chaetoceros muelleri*) from the Horn Point Oyster Hatchery (Cambridge, MD) were added to beakers of artificial seawater at a salinity of 2.5. Algae were added to each 800 mL beaker in 1 mL increments until the FluoroSense™ Handheld Fluorometer (model # 2860-000-C, Turner Designs, San Jose, CA) reading reached just below the maximum detection limit (199 μg/L). Subsamples of water were collected from the beaker

after each incremental algae addition and algal cells were counted (cells/ml) in triplicate on a Levy Improved Neubauer hemocytometer (Hausser Scientific, PA) using an Olympus BX41 microscope at 20X magnification. To determine the statistical relationship between chlorophyll concentration (µg/L) measured by the fluorometer and the mean cell concentration (cells/mL) measured from triplicate hemocytometer counts, standard curves were calculated using ordinary least squares regressions through the origin using the *stats* package (version 4.0.2) in the R statistical software (version 3.6.1; R Core Team, 2020). Separate serial dilutions and standard curves were calculated for each feeding day, except for the last day (June 20th, Figure 1). The relationship between chlorophyll (µg/L) and algal concentration (cells/mL) 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) feeding day (Figure 1).

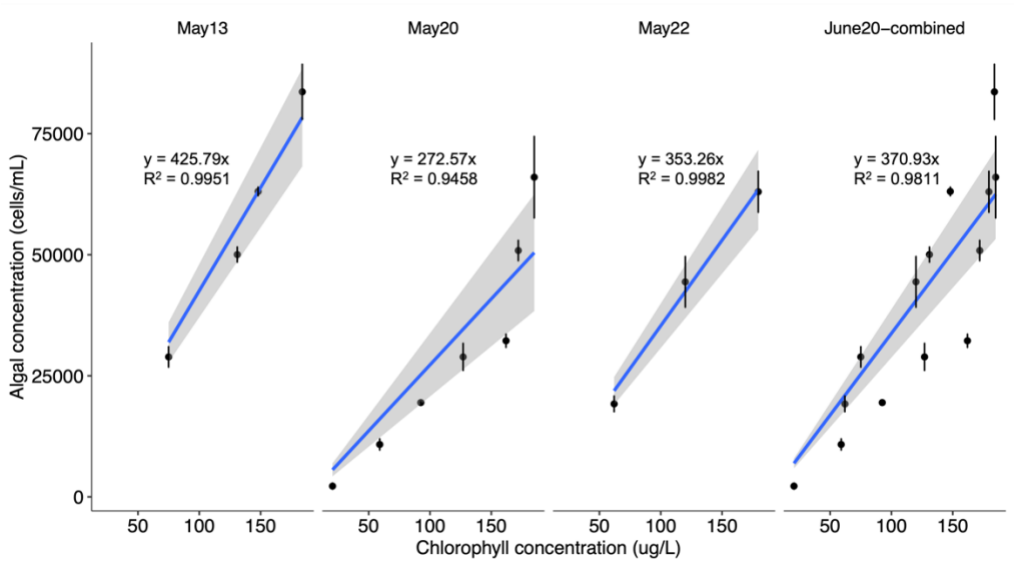

Figure 1. Relationship between algal (average of triplicate hand counts; cells/mL) and chlorophyll concentration (ug/L) for the serial dilutions from the feeding analysis trials. The equations determined from the ordinary least squares regression lines (in plot) were forced through the origin and the 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 SEM for the triplicate hand counts.

After the correct volume of algae was added to each beaker (~ 9 – 11 mL; ~75,000 cells/mL), each individual oyster was removed from the chronic 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 chronic 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. Triplicate beakers with no oysters were used as a control for each experimentation day to account for any cell sinking or sticking to the sides of the beaker. We also tested control beakers with empty oyster shell to assess the effect of shell presence on algae concentration, but no differences in algae depletion was observed. Thus, we report data only from the control beakers with no shell. Algal removal rate was assessed for individuals (N = 3) from three of the half-sibling families and from the wild population in the control tank (salinity ~6). All individuals depleted all the algae within the first 4 hours of testing and feeding analysis on ambient individuals was not replicated.

Statistical analysis of feeding rate experiment

Fluorometer readings (μg/L) were converted to cellular abundance estimates (cells/mL) using the standard curves described above. For each individual oyster, we calculated three

metrics of algal removal for subsequent analysis: average feeding rate (FR_{avg}), maximum algal removal rate (r_{max}), and time to 50% algal depletion (D_{50}). We then took an average of these three metrics for all individuals within a family to investigate relationships between feeding rate and survival in the chronic low salinity challenge.

To determine the average feeding rate for each individual, feeding rates (FR, cells/mL/hr) were calculated at each time interval (i.e. 0–3 hours, 3–6 hours, etc.) and rates were normalized to individual shell height (mm; Coughlan, 1969; McFarland et al., 2013; Riisgård, 1988):

$$FR = \left(\frac{V}{t} * \left(\ln\left(\frac{C_0}{C_t}\right) - A\right) \right) / L$$

where V= volume of water in liters, t is elapsed time in hours, C_0 is the initial concentration (cells/mL), C_t the algal concentration at the given sampling time, A is the average algal cell loss across the three control jars for the specified time interval (i.e. $\ln(\frac{A_0}{A_t})$), and L is the shell height (mm) of each individual. Normalization was conducted using shell height because there were not enough oysters for destructive, dry weight sampling. An average feeding rate was calculated for each individual by averaging the series of feeding rates (FR_{avg} = average feeding rate). Feeding rates for time intervals where algae was already depleted, and therefore fluorescence was not measured, were excluded from the overall average.

Locally estimated scatterplot smoothing (LOESS) splines were used to determine two additional feeding rate 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) present over the 24-hour period using the *stats* package (version 4.0.2) in the R statistical software (version 3.6.1; R Core 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. Maximum algal removal rate for each individual was estimated as the derivative at the steepest part of the LOESS curve. The absolute value of r_{max} was used to make this value positive (i.e. the slope or rate of algal depletion is a negative value) and individual D_{50} and r_{max} were divided by individual shell height for size normalization.

A family average was taken for each of the three algal depletion metrics. Ordinary least square regressions using the *glm* package (*stats* version 4.0.2, R version 3.6.1; R Core Team, 2020) were performed to determine if family FR_{avg}, family D_{50}, and family r_{max} were predictive of family cumulative survival in the chronic (6-month) low salinity challenge. Family cumulative survival (proportion between 0 and 1) was logit transformed before regression analyses to make the variable normally distributed. Lastly, paired t-tests were run on the three feeding metrics (FR_{avg}, D_{50}, and r_{max}) for individuals (N = 4) that were repeated on multiple days to assess the effect of experimental day on the measured metric (i.e. effect of varying temperature and other experimental design factors).

Results

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

Differential mortality was observed between the 51 half-sibling families during both challenges at a salinity of ~2.5 and at a temperature of 27 °C (short-term challenge) and at a fluctuating temperature (chronic challenge). Before individuals were moved from the chronic to the short-term challenge, cumulative mortality for all families was < 4%, except for one family (family 83), where mortality was 8.5%. Mortality in the short-term challenge 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. 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 chronic 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 2). Temperature was greater than 27°C for 27 days, beginning on day 86, before the second mortality spike was observed on day 112.

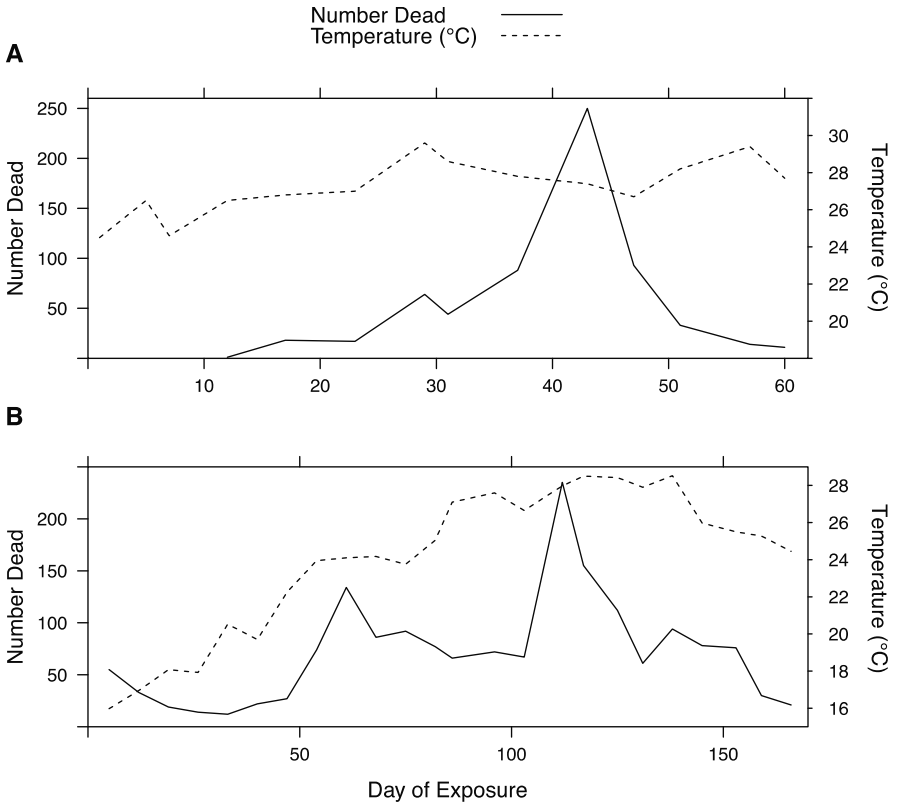

Figure 2. Number of dead individuals throughout the two low salinity challenges. A) Daily mortality (solid black line) and temperature (°C, dotted black line) during the A) short-term, 60-day challenge, and B) chronic, 168-day challenge.

A two-way ANOVA revealed no statistically significant interaction between the effects of family and sampling month on individual height (ANOVA, $F_{300,17328} = 1.062$, $p = 0.221$). Main effect analysis showed a statistically significant effect of both family (ANOVA, $F_{50, 17328} = 98.12$, $p < 0.001$) and sampling month (ANOVA, $F_{6, 17328} = 56.80$, $p < 0.001$) on individual height, likely due to the significant differences in family height before the chronic challenge began ($F_{50,2493} = 18.12$, $p < 0.001$). A two-way ANOVA revealed a statistically significant interaction between family and sampling month on individual height ($F_{30,1008} = 2.4465$, $p =< 0.001$) in the control tank at ambient salinity. There was no correlation between mean family height pre-exposure (March, mm) and family cumulative survival in the chronic challenge ($r_s = -0.168$, $p = 0.236$). Families in the control tank grew an average of 26.09 mm over 6 months at ambient conditions, while families in the chronic challenge shrunk an average of 3.025 mm.

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

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

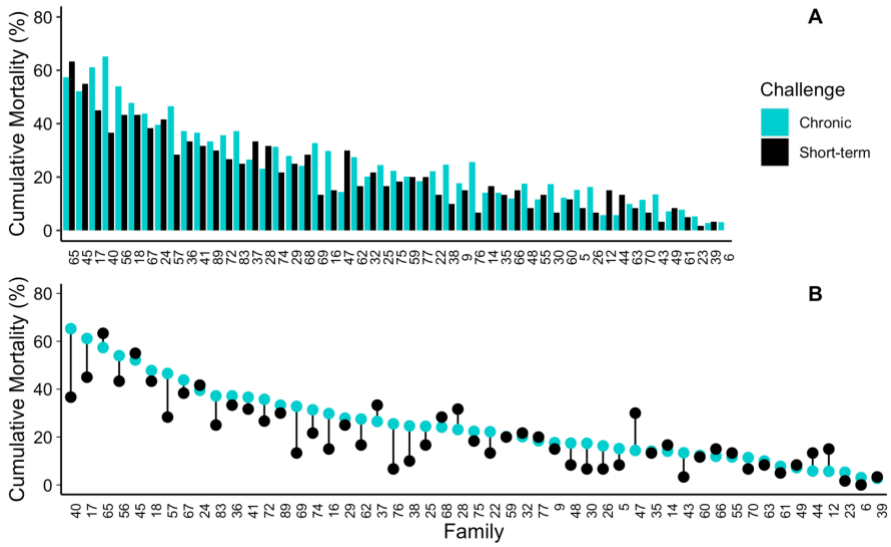

Figure 3. Family mortality during the two low salinity challenges. Cumulative family mortality (%) from A) the short-term (black) and the chronic challenge (aqua), and B) a lollipop plot depicting similar family mortality across the two lab challenges.

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 for the short-term challenge, and 0.4093 ± 0.036 for the chronic 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, Table 1).

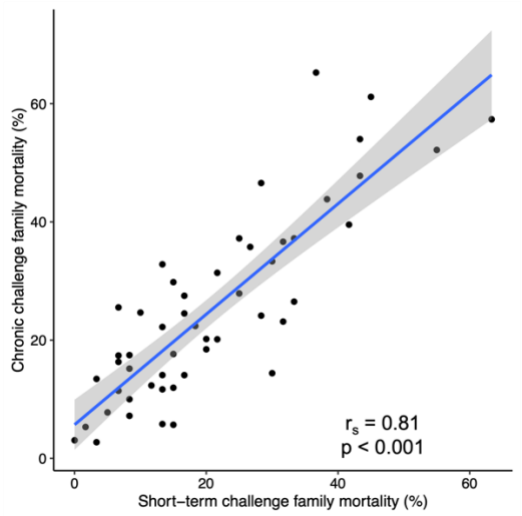

Figure 4. Scatter plot displaying correlation in cumulative mean family mortality (%) between the chronic and short-term challenges. Spearman rank correlation coefficient (r_s) and significance value is displayed in the bottom right, and shading represents the 95% confidence interval for the ordinary 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
Chronic	0.4093 ± 0.036	0.89 ± 0.07	0.814
Short-term	0.3505 ± 0.026		

Feeding rate analysis

Most oysters across the 31 families (FR_{avg} > 0 for 87% of oysters examined) fed at a salinity of 2.5, reducing the concentration of algae in the experimental beaker over the 24-hour sampling period. Oysters that were measured (repeatedly) across multiple experimental days (N = 4) had very similar feeding rate metrics, including r_{max} (paired t-test, t(4) = 0.523, p > 0.1), FR_{avg} (paired t-test, t(4) = -1.02, p > 0.1), and D_{50} (paired t-test, t(4) = 2.14, p = 0.1). The rate of decline in phytoplankton concentration (cells/mL) over time generally demonstrated a sigmoidal

relationship, in which algal concentration was high and unchanged initially (oysters slow to feed/filter during the first few time points) before dropping over time as oysters filtered and removed algae from the water column (Figure 5). However, the rate at which cells were removed varied greatly among individual oysters. Individuals from families with high cumulative survival during chronic low salinity exposure removed algae over a relatively shorter duration of time and almost always depleted the entire concentration of starting algae (green curves; Figure 5). In contrast, individuals from families with low cumulative survival in the challenge removed a smaller portion of the initial concentration, at lower rates on average, and some families failed to reach 50% algae depletion (red curves; Figure 5).

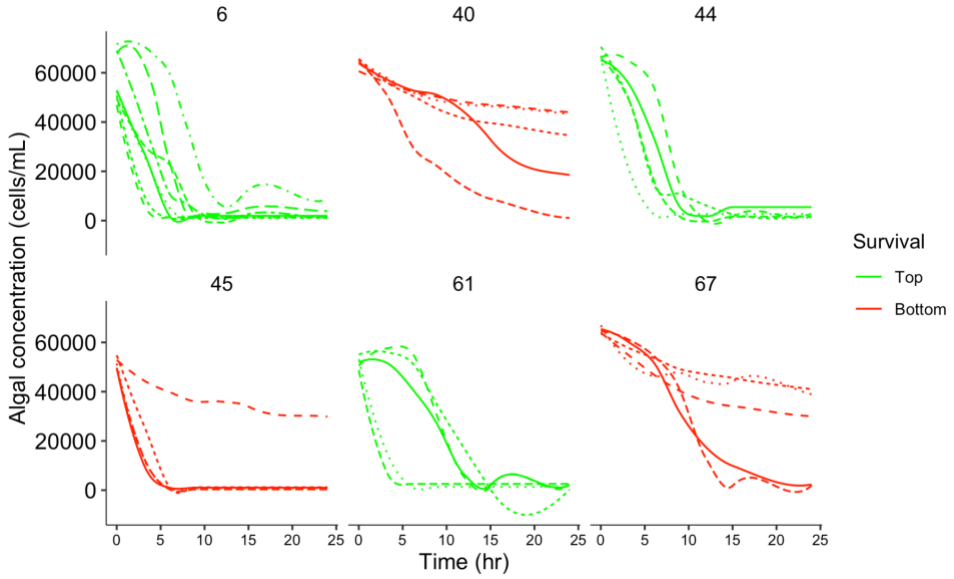

Figure 5. Algae concentration (cells/mL) over 24 hours for 6 families sampled for algal removal at a salinity of 2.5. Individual replicates are depicted by different line types and grouped together by family. Lines are color-coded based on the relative ranking of survival across all families, red indicating lowest survival (bottom ten surviving families) and green indicating highest ranking survival (top ten surviving families).

Family average feeding rate normalized for individual height (FR_{avg}; cells/mL/hr/mm) ranged from 0.0893 – 14.69 cells/mL/hr/mm, with an average family FR_{avg} of 4.710 cells/mL/hr/mm. The association between family FR_{avg} and family mean survival (logit transformed) in the chronic (6-month) low salinity challenge was marginally significant at the alpha 0.10 level ($p = 0.08$; Figure 6A). Family maximum algae removal rate normalized for individual height (r_{max}; cells/hr/mm) ranged from 76.29 – 290.0 cells/hr/mm with an average family r_{max} of 172.7. Family r_{max} was significantly associated with survival in the chronic challenge ($p = 0.035$, Figure 6B). While both associations explained only a small proportion of the variation (10 – 14%), results indicate that both a higher family average feeding rate and a higher maximum algal removal rate are associated with higher family survival during low salinity exposure (Figure 6A, B). Family average time to deplete 50% of the starting algae concertation normalized for individual height (D_{50}; h/mm) ranged from 0.0632 to 0.4948 hr/mm with an average family D_{50} 0.2597 hr/mm. The association between family D_{50} and family mean survival was just above the alpha 0.10 significance level ($p = 0.15$) and explained a small portion of variation (7%; Figure 6C). A total of 35 (19.7%) individuals labeled after the feeding rate assessment died after being returned to the chronic challenge. Individuals that died had an average FR_{avg} of 3.547 cells/mL/hr/mm, an average r_{max} of 128.1 cells/hr/mm, and an average c_{50} of 0.3135 hr/mm. Thirteen oysters that died (37%) never reached the 50% depletion mark. Feeding metrics were highly associated to one another (all p-values < 0.01 and R^2 values ranged from 0.22 – 0.54; Figure 6D - F), indicating they represent similar feeding phenotypes.

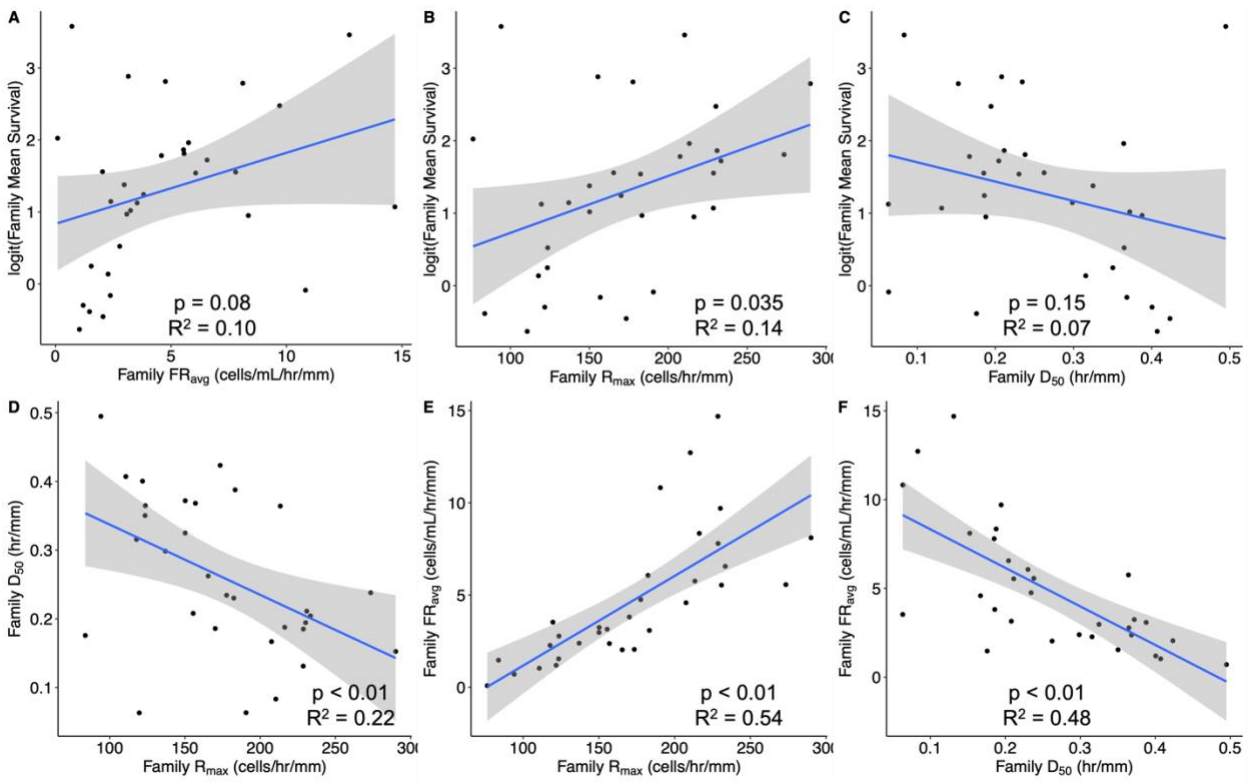

Figure 6. Scatter plots and ordinary least square regression lines of family mean survival (logit transformed) against A) family average feeding rate (FR_{avg}) (cells/mL/hr/mm), B) family average maximum algal removal rate (r_{max}) (cells/hr/mm), and C) average time for each family to deplete 50% of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

Discussion

A short-term (2 month) and chronic (6 month) challenge at extreme low salinity (<3) was conducted to investigate the effect of challenge duration and varying temperature on oyster low salinity survival. During the 6-month challenge, temperature was adjusted daily to mimic the natural, seasonal variation in temperature experienced from Spring to Fall in the Choptank River,

MD. Furthermore, the chronic challenge allowed for the decoupling of the two stressors (high temperature and low salinity), as extreme low salinity (< 3) and high temperature (27°C) were explicitly tested during the short-term challenge. Similar to results from previous extreme low salinity challenges (e.g. McCarty et al., 2020), mortality varied among families in the two challenges (but was highly correlated between short and long challenges for a given family) and narrow-sense heritability estimates for low salinity survival were moderate and significantly different from zero. The removal of algae from a 1 Liter beaker over a 24-hour period varied among individuals and families when exposed to an extreme low salinity (< 3) and the three metrics used to quantify algae removal (family average feeding rate: FR_{avg}, family maximum algal removal rate: r_{max}, and family time to half: D_{50}) were marginally significant when associated to family survival in the chronic low salinity exposure. Below, we discuss in detail the biological and practical implications of the chronic versus short-term challenge results. We also draw inferences from the feeding experiment work and discuss the potential of a feeding phenotype for use in future low salinity breeding.

Fine-tuning a progeny test for survival in extreme low salinity

A primary goal of this study was to assess how a longer-term low salinity challenge with a more natural (ambient) temperature regime would impact results in terms of overall mortality. Family mortality was highly correlated across the two challenges (> 0.8 for both the phenotypic and genetic correlations) and the rank of families was similar between the challenges (Figure 3B & Figure 4). Additionally, heritability was similar and moderate for both experiments (chronic h^2 = 0.4, short-term h^2 = 0.35). The cumulative mortality observed during our two challenges (23 and 25.2%) and the two heritability estimates (h^2 = 0.35 and 0.4) are very similar to values previously observed during a low salinity 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.

Reduced cumulative mortality was observed in the chronic challenge compared to the previous low salinity challenges conducted in the summer. In the chronic challenge, both the magnitude of mortality during the peak in July and the overall cumulative mortality (25.8%) were not nearly as large as previously reported during mid-summer challenges (53% cumulative mortality across families in July-August, McCarty et al., 2020; nearly 100% cumulative mortality in F_2 families in June-July, McCarty et al., 2021). The reduction in mortality in the chronic challenge compared to previous low salinity challenges conducted in our lab, may be a result of the low ambient temperature (7.9°C) when the challenge began on April 1st. In previous challenges (McCarty et al., 2021, 2020), oyster gonads were likely mature or maturing, and spawning may have already been occurring, based on the high ambient temperatures when oysters entered the challenges (~24°C on May 28 and ~26°C on July 17 in 2018, data courtesy of Horn Point Oyster Hatchery). In the chronic challenge, oysters should have been undergoing 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 stunted gonad development and arrested gametogenesis would free up the reserves and energy required to endure the stress associated with low salinity exposure. Conversely, lower cumulative mortality in the chronic challenge may

simply be a result of the gradual increase in temperature opposed to a 2-day step up to stressful temperature levels (27°C).

Oyster size-class affects the time it takes for mortality to occur in the low salinity challenge. The short-term and chronic challenge, along with previous low salinity challenges (McCarty et al., 2020, 2021), used oysters sourced from the ABC breeding family lines at VIMS (Allen et al., 2021). However, the oysters used in this study (short-term and chronic challenge) were smaller and younger (< 40 mm, 1-yr old) compared to the adult individuals (>80 mm) used previously (McCarty et al., 2020, 2021). Mortality peaked after 6 weeks at 27°C in the short-term challenge (more comparable to previous low salinity challenges: constant temperature of 27°C and 1-2 month exposure duration), while peak mortality occurred one week after exposure using >80 mm adults in previous studies (McCarty et al., 2020, 2021). 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, 2021). 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; suggested in McCarty et al., 2020; Rybovich et al., 2016). Smaller oysters are suggested to be more tolerant to stressful conditions because maintenance costs scale with body volume, where a larger individual requires more energy to maintain somatic and gonadal function (DEB theory, Kooijman, 2010). Thus, at a salinity <3 and temperature of 27°C, 1-year old oysters (< 40 mm) take at least 6 times as long for appreciable mortality to occur, which is important to consider when designing future progeny tests.

It is worth investigating the phenotypic correlation in family survival at different life stages (e.g. spat/seed, juvenile, adult) to determine which life stage is preferred for progeny testing and for improving survival in low salinity in general. Previous studies investigating growth-related and disease-resistant traits in bivalve species have been conducted on individuals at four (Gutierrez et al., 2020), six (Gutierrez et al., 2018b), nine (Van Sang et al., 2019; Vu et al., 2021), 15 (Dou et al., 2016), and 24 months (Yangfan Wang et al., 2018), but the effect of age on those traits was not examined. For the eastern oyster, genetic correlations for growth-related traits measured at 1.5 and 2.5 years old were high (total weight, width index, height index were > 0.96, meat yield > 0.8, Allen et al., 2021), and correlations were high but slightly lower for survival in a low salinity of 6 - 15 (r_G = 0.72, Allen et al., 2021). This suggests, at least for these yield traits, 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 get measurable mortality (> 80 mm, 2-4 weeks, McCarty et al., 2020, 2021), but maintaining animals until, at least, 2 years old before testing can be costly from a husbandry standpoint. Moreover, low salinity survival may differ between spat/seed and adult oysters, which would suggest treating these as two separate traits with separate progeny tests. Whether or not similar correlations among age classes would exist for low salinity survival remains to be examined.

Measuring feeding rate as a possible indicator of low salinity survival

One feeding metric (family maximum algal removal rate, r_{max}) was significantly associated to family survival in the chronic exposure challenge, while the other two (family average feeding rate, FR_{avg}, and family average time to 50% depletion, D_{50}) were marginally associated. The general feeding trends from this study suggest that families with oysters that begin feeding sooner and deplete the available algae more quickly were more tolerant of extreme low salinity conditions (i.e. higher survival). Based on these results, it appears that both family r_{max} (derivative of the steepest part of the LOESS curve) and family FR_{avg} are higher for individuals from families that are more tolerant to extreme low salinity. Similarly, individuals from families with high survival in the chronic challenge had a lower D_{50}. While these results suggest that feeding efficiency, in terms of the onset and rate of feeding, is associated with higher tolerance to extreme low salinity, the associations were weak (Figure 6A-C). The weak relationships could be due to the relatively small number of individuals sampled (N = 178). There was also a great deal of variation among replicates (individuals) within each family, which also could have reduced the strength of the relationships. Feeding and clearance rates are influenced by many different factors and have proven to be highly variable in shellfish (e.g. Cranford et al., 2011, 2005; Grizzle et al., 2008; Li et al., 2012), making it inherently difficult to measure and quantify these associations. Future experiments would need to be conducted with larger sample sizes and more replicates per family to ensure that the results obtained are repeatable. Individuals sampled on multiple days, and at slightly different temperatures (20.7°C - 24.7°C), had very similar (consistent) performance in feeding metrics, indicating that feeding trials can be conducted across multiple days throughout the summer to include more individuals and families.

Given the moderate but significant relationship between feeding and survival during the challenges, it is interesting to consider how reduced feeding might be impacting the ability of oysters to survive during these challenges. Oysters feed by capturing suspended particles on the ciliated structures of their ctenidium (or gills) as water is pumped into the inhalant siphon, through the spaces between the gill filaments, and then out the exhalent siphon (Ward and Shumway, 2004). If oysters are not removing algae, this would produce a feeding rate slope close to 0 (i.e. horizontal line), which was often observed for individuals with poor survival in the chronic exposure (e.g. Figure 5). Reduced feeding may result from differences in the structure of the gills and/or cilia or from reductions in water flow due to active reduction in cilia beating. Water pumping is also affected by other musculature structures that control shell gape, exhalent siphon area, and interfilamentary distance to the gill (mechansisms of feeding reviewed in Cranford et al., 2011). Oysters can ultimately impact their food acquisition by controlling each of these mechanisms independently and to variable degrees depending on environmental stimuli (reviewed in Cranford et al., 2011; Maire et al., 2007; Newell et al., 2001). It may be worth investigating overall pumping rate or looking at each of these mechanisms in more detail to determine why some animals may display reduced feeding.

During exposure to extreme low salinity, oysters may be open but not actively filtering particles from the water. We previously determined that individuals, even from low performing families, open during the first week of exposure to a low salinity challenge to osmoconform (McCarty et al., 2020), but they may not be feeding. In bivalves, gape/valve opening controls water flow into an organism for filtration and gas exchange, but valve opening has been shown to poorly correlate with clearance and feeding rates (e.g. Dolmer, 2000; Frank et al., 2007; Newell et al., 2001), suggesting that clearance/feeding is independent of gas exchange.

Monitoring valve movement coupled with observations of feces/pseudofeces production could determine if individuals are simply closed, open and filtering, or open and unable to filter. Conversely, the variation in feeding ability at low salinity may be a consequence, and not a cause, of variable low salinity survival. Forty percent of individuals that died after being returned to the chronic exposure never reached the 50% algae depletion mark during their feeding trials. Thus, the observed decreases in feeding may be a result or consequence of poor health/condition of oysters (oysters starting to die) and not predictive or causative of variable survival in stressful low salinities.

Coupling metabolic physiology with clearance/feeding rates may explain the observed differences in family mortality. Families with low feeding rates and high metabolic rates, typically measured as oxygen consumption and referred to as a respiration rate (Bayne, 2017), may have higher mortality because they are not able to meet high metabolic demands. While low salinity has overall negative impacts on general oyster physiology (respiration, clearance, and feeding; e.g. Casas et al., 2018a, 2018b; La Peyre et al., 2020; Loosanoff, 1952; Shumway and Koehn, 1982), differences in basal metabolic rates have been observed for distinct populations of oysters (e.g. Méthé et al., 2020; Pernet et al., 2008). Families with lower resting metabolic rates may be better able to cope energetically during stressful low salinities compared to families that have higher resting metabolic rates. Lower metabolic rates have been associated with higher growth rates and scope for growth in *Crassostrea virginica* (Pernet et al., 2008) and have also been associated with increased resilience to summer mortality syndrome in the blue mussel, *Mytilus edulis* (Tremblay et al., 1998). Thus, lower metabolic rates may be related to increased fitness in oysters (Méthé et al., 2020) and whether a genetic component underlie these metabolic differences remains an interesting question worthy of future attention.

Conclusion

This work, along with previous experimental challenges (McCarty et al., 2021, 2020), provide insight into the effect of temperature, exposure duration, and oyster size/age on mortality during extreme low salinity (< 3). Based on our results, measuring algae concentration using a handheld fluorometer appears to provide an accurate tool for rapid assessment of algal removal in vitro. Modest associations between algae removal metrics and survival in extreme low salinity suggest that algae removal, measured as a feeding rate and quantified using smoothing splines, could be suggestive of survival in a low salinity progeny test. If this test did reliably predict salinity survival, then sensibly, a progeny test could be developed to assess feeding behavior at low salinity on a large number of individuals over the course of just days with minimal experimental setup and maintenance, and breeding families could be selected based on average family algae removal performance. This test could be especially valuable when assessing low salinity survival in younger individuals, where mortality may take longer (~6 weeks) to occur. However, our results are preliminary and this needs to be investigated in more detail (larger sample size and more replicates per family). Based on our results, when using the top performing families (low mortality in an extreme low salinity challenges), oyster aquaculture operations can persist in areas that frequently experience periods of extreme low salinity.

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Chapter 4: Genome-wide analysis of acute low salinity tolerance in the eastern oyster *Crassostrea virginica* and potential of genomic selection for trait improvement

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Abstract

As the global demand for seafood increases, research into the genetic basis of traits that can increase aquaculture production is critical. The eastern oyster (*Crassostrea virginica*) is an important aquaculture species along the Atlantic and Gulf Coasts of the United States, but increases in heavy rainfall events expose oysters to acute low salinity conditions, which negatively impact production. Low salinity survival is known to be a moderately heritable trait, but the genetic architecture underlying this trait is still poorly understood. In this study, we used ddRAD sequencing to generate genome-wide single nucleotide polymorphism (SNP) data for four F_2 families to investigate the genomic regions associated with survival in extreme low salinity (< 3). SNP data were also used to assess the feasibility of genomic selection for improving this trait. Quantitative trait locus (QTL) mapping and combined linkage disequilibrium analysis revealed significant QTL on eastern oyster chromosome 1 and 7 underlying both survival and day to death in a 36-day experimental challenge. Significant QTL were located in genes related to DNA/RNA function and repair, ion binding and membrane transport, and general response to stress. Genomic selection was investigated using Bayesian linear regression models and prediction accuracies ranged from 0.48 – 0.57. Genomic prediction accuracies were largest using the BayesB prior and prediction accuracies did not substantially decrease when SNPs located within the QTL region on Chr1 were removed, suggesting that this

trait is controlled by many genes of small effect. Our results suggest that genomic selection will likely be a viable option for improvement of survival in extreme low salinity.

Introduction

Food insecurity is a global crisis that affects more than a quarter of our population worldwide, but aquaculture provides hope for meeting increasing food demands (FAO et al., 2019). Globally, aquaculture has out-produced capture fisheries for a decade (FAO, 2020b), and, as of 2018, is the fastest growing sector of food production worldwide (FAO, 2018b). Marine and coastal aquaculture, specifically, comprised 36% of total aquaculture production in 2016, and nearly 60% of this production came from marine bivalve aquaculture (FAO, 2018b). Over the last 20 years, the Chesapeake Bay, located on the eastern seaboard of the United States, has seen substantial increases in eastern oyster *(Crassostrea virginica)* production from aquaculture (Hudson, 2018; Senten, Engle, Parker, & Webster, 2019). As of 2019, there was an estimated 429 total leases comprising 6,930 total acres for eastern oyster aquaculture in the Maryland-portion of the Bay (van Senten et al., 2019). Oyster harvest increased 115% from 2010 – 2018, and the Maryland shellfish industry was estimated to have an economic impact of over $8 million (van Senten et al., 2019). The oyster industry provides a substantial input to the economy of Maryland, and also provides valuable employment opportunities in coastal areas where industry is limited.

While eastern oyster aquaculture is expanding in the Chesapeake Bay, the highly variable salinity gradient is one of the most prominent environmental factors hindering production for aquaculture operations. Harvest numbers, economic input, and employment associated with the shellfish aquaculture sector in Maryland were substantially lower in 2018 compared to 2017, primarily due to the abnormally low salinity in the Bay resulting from the large inflow of

freshwater from heavy rainfall (NOAA National Centers for Environmental information, n.d.; van Senten et al., 2019). Oyster aquaculture in the upper Bay is periodically faced with the threat of extreme low salinity (< 3) resulting from heavy rainfall associated with large storm events. Large mortality events from extreme low salinity (< 5) have been observed in estuarine and coastal systems globally (reviewed in Du et al., 2021), such as in the Chesapeake Bay (Andrews et al., 1959; Engle, 1946; Southworth et al., 2017), in the Gulf of Mexico (Butler, 1952a, 1949; Du et al., 2021; Gledhill et al., 2020), and in northern California (Cheng et al., 2015). A lower ideal salinity (~ 9 – 16) has recently been proposed for eastern oyster populations in Louisianna estuaries where freshwater input dominates the hydrodynamics of the system (e.g. La Peyre *et al.* 2016; Rybovich *et al.* 2016; Lowe *et al.* 2017), which is most likely the case for many locations in the northern portion of the Chesapeake Bay. Within the Chesapeake Bay, a "low salinity" oyster line currently exists (salinity ~6-15; Allen, Small, & Kube, 2021), but, growth and survival at low salinity (5-10) is arguably different than growth and survival at extreme low salinity (< 3) (McCarty et al., 2020).

Survival under salinity stress was recently determined to be a heritable trait in the eastern oyster. Survival in high salinity (~15 – 23) is a distinct trait from survival in low salinity (~6 – 15) (Allen et al., 2021), and survival in both low salinity (~6 – 15) and in extreme low salinity (< 3) have proven to be moderately heritable (salinity ~6 – 15 $h^2 = 0.34$; salinity < 3 $h^2 = \sim 0.4$; McCarty et al., 2020). However, genomic (marker-based) analyses of low salinity tolerance in oysters have not been conducted, and knowledge of the genetic architecture of a trait is important when initiating, or establishing, an effective breeding program. Previous genomic investigations of aquaculture traits in the eastern oyster have been focused primarily on resistance to *Perkinsus marinus*, the causative agent of Dermo disease (Yu and Guo, 2006). In other aquaculture species,

identified quantitative trait loci (QTL) have successfully been incorporated into marker-assisted selection (MAS) programs, for example, for disease resistance in Japanese flounder (Fuji et al., 2007), Atlantic salmon (Houston et al., 2008; Moen et al., 2015, 2009), and Rainbow trout (Liu et al., 2018). However, MAS is typically ineffective for most production traits due to their highly polygenic nature, meaning the trait is controlled by many loci of small effect (Houston et al., 2020; Zenger et al., 2019). On the other hand, genomic selection (GS), or the selection of individuals based on the combined genetic effect of all relevant genome-wide polymorphisms (Meuwissen et al., 2001) may be more effective for polygenic traits and produces higher accuracies of selection and higher rates of genetic gain compared to traditional, exclusively performance and family-based, selective breeding programs (Houston et al., 2020; Ødegård et al., 2014; Zenger et al., 2019). Implementation of genomic selection for aquaculture species was once limited to well-studied species such as Atlantic salmon and rainbow trout (Ødegård et al., 2014; Vallejo et al., 2017; Zenger et al., 2019), but recent advances in genomic technology and resources have increased the accessibility of GS for many aquaculture species (Houston et al., 2020). The effectiveness of MAS or GS for advancing breeding depends on the genetic architecture of a trait, which is currently unknown for extreme low salinity survival in the eastern oyster.

In this study, we performed QTL mapping and combined linkage disequilibrium analyses in four F_2 oyster families, originating from a low and high salinity line, exposed to an acute low salinity experimental challenge (< 3). Tissue was collected from all individuals, both dead and alive, and genome-wide single nucleotide polymorphisms (SNPs) generated with ddRADseq (Peterson *et al.* 2012) were used to investigate genomic regions associated with survival and day to death. The potential for using genomic selection to advance breeding of low salinity survival

was also investigated by calculating genomic prediction accuracies via cross validation for several Bayesian linear regression models. This work provides initial insight into the genetic architecture underlying survival in acute low salinity (< 3) for the eastern oyster and will help determine whether MAS or GS may provide a better approach for selective breeding of this trait.

Materials and methods

F_2 breeding design

In 2014, 10 F_1 hybrid families were generated by the Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science from crosses between individuals from the low salinity and high salinity family breeding lines (Allen et al., 2021). In 2015, 8 F_2 families were made by ABC from full-sibling pair-matings within the F_1 families, and all larvae and seed were reared following standard VIMS protocols (Allen et al., 2021). Seed reached ¼ to ½ inch by September and were then transferred to the Horn Point Laboratory (HPL; MD, USA). Once at Horn Point Laboratory, seed were overwintered in the HPL boat basin until March of 2016 when they were put into 3/16-inch vexar mesh bags in a rack and bag setup on the intertidal beach at the Horn Point demonstration farm. Seed were grown in a rack and bag system and checked monthly for biofouling from March – November 2016. From 2016 – 2018, oysters were moved to the HPL boat basin for overwintering from November – March, and then returned to the intertidal demonstration farm from March – November. In March 2018, oysters were transferred to SEAPA® baskets and deployed on an Australian Longline system in the intertidal zone of the demonstration farm at the Horn Point Laboratory until being brought into the laboratory in May 2018 for experimentation. Oysters were ~ 3 years old and averaged 92.37 mm ± 0.44 standard error of the mean (SEM) when experimentation began on May 28, 2018.

Acute low salinity experimental challenge

Oysters from the eight F_2 families (N = 70 – 140 oysters per family) were randomly divided into equal-sized replicate plastic baskets depending on total number of oysters for each family. Replicate baskets were secured to the bottom of custom-made Taylor floats and submerged in 6-ft diameter tanks (~1800 L) located indoors at the Horn Point Laboratory in Cambridge, Maryland, USA. Oysters were exposed to acute low salinity (< 3) following a very similar experimental design to McCarty et al. (2020): a 1-week acclimation period at ambient conditions followed by a 2-day salinity step-down and simultaneous temperature increase. Continuously flowing Choptank River water (salinity ~7-11) and oxygenated, heated well water (salinity 0) were mixed by hand to maintain salinity 2.3 ± 0.13 SEM and temperature 26.9°C ± 0.07 SEM for 36 days, from May 28 to July 5. A salinity lower than a prior challenge was chosen in hopes of increasing mortality during the experimental timeframe, as only 23% cumulative mortality was observed previously using half-sibling families (experiment 1:April 5 - May 7, McCarty et al., 2020). Salinity, temperature, and dissolved oxygen were recorded daily with a YSI-85 handheld multimeter (YSI Incorporated, Yellow Springs, OH, USA). Feeding was supplemented daily with Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA) at 1.5% total dry tissue biomass and with 3 L of live, cultured phytoplankton from the Horn Point Laboratory Oyster Hatchery. Individual mortality was assessed daily by checking for gaping individuals (McCarty et al., 2020), and survival and day of death was recorded for every individual. Adductor muscle was sampled and preserved in 95% ethanol when individuals died, and for all individuals remaining alive at the end the experiment.

Library preparation, sequence mapping, and SNP filtering

Four families (11, 43, 22, and 65) were chosen for downstream analysis because they had the largest sample size per family and had the largest range in mortality across the 36-day challenge period (i.e., individual death occurred over many experimental days). A total of 132, 114, 111, and 106 individuals were analyzed for family 11, 43, 22, and 65, respectively. DNA was extracted from a total of 471 tissue samples, 463 samples from the challenge individuals plus the dams and sires of the four F_2 families, using the E.Z.N.A Tissue Extraction Kit (Omega Bio-tek, Norcoss, GA) following the protocol for preserved animal tissue. Following extraction, DNA concentration was quantified for each sample using a Qubit Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Genome-wide SNPs were generated using double digest restriction association DNA (ddRAD) sequencing techniques outlined by Peterson and colleagues (Peterson et al., 2012). Following digestion with EcoRI and SphI (the "flex-set"), barcoded adapters (1-48) were ligated to digested DNA for each individual separately in a microplate format. Barcoded samples were pooled, and size selection was performed using Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN) to select fragments 300-800 bp in length. Size-selected libraries were amplified using the Phusion High-Fidelity PCR Kit (New England BioLabs, Ipswich, MA) and run for 10-12 cycles with a specific indexed primer appropriate for standard Illumina multiplexed paired-end sequencing. Three libraries, each comprising 250-275 individuals, were sent to GeneWiz (South Plainfield, NJ, USA) for next generation single-index sequencing on three Illumina HiSeq 2x150 bp sequencing lanes with 15% PhiX spike-in. For each F_2 family, 5% of the individuals were duplicated to calculate genotype error rate at each locus. GeneWiz demultiplexed libraries based on Illumina indexes, and libraries were further demultiplexed into individual barcoded libraries and renamed

using the *process_radtags* function from the Ddocent pipeline (Puritz et al., 2014). All reads from all individuals in the four F_2 families were grouped together for downstream analysis.

Reads were trimmed and aligned to the eastern oyster reference genome C_virginica-3.0 (GenBank accession GCA_002022765.4; Gómez-Chiarri, Warren, Guo, & Proestou, 2015) using the dDocent pipeline with parameters A (match score), B (mismatch score), and O (gap penalty) set to 1, 3, and 5 respectively, which have proven to be more appropriate for marine species (Dimens et al., 2019; Puritz et al., 2014). After alignment, FreeBayes (version 1.2.0-dirty, Garrison & Marth, 2012) was used for SNP discovery and genotype calling, and SNPs were filtered following the dDocent step-wise filtering pipeline for missing data, genotype depth, locus quality score, minor allele frequency, and genotype call depth (Puritz et al., 2014). Individuals with more than 50% missing data were removed, and retained SNPs were present in 90% of individuals, had a minimum read depth of 20 sequences per genotype, a minimum sequence quality score of 30, and a minimum minor allele frequency of 0.05. *dDocent_filters* was used to further filter SNPs based on allele balance, quality/depth ratio, mapping quality ratio of reference and alternate alleles, properly paired status, strand representation, and maximum depth using suggested parameters. Polymorphisms were decomposed and indels were removed using v*cfallelicprimitives*. Lastly, SNPs were tested for Hardy-Weinberg equilibrium using a previously developed script (https://github.com/jpuritz/dDocent/raw/master/scripts/filter_hwe_by_pop.pl) and SNPs falling below a p-value of 0.001 in one or more families were removed. Parents for one of the families (22) had substantial missing genotype data, so the initial individual missingness filter threshold was relaxed for this family only (individuals with <65% missingness retained). All subsequent filtering steps were identical between family 22 and the other families (e.g. SNP call rate >90%,

allelic imbalance, max depth, paired status, etc.). The combined linkage disequilibrium analysis (all families combined) was performed on the dataset with initial missingness set at <50% (see below). Genotype error rate (%) was calculated for 21 DNA samples with duplicate RAD library preps (same DNA different barcode) as the cumulative number of mismatches between duplicate genotypes at each SNP divided by the total number of genotypes tested, not including SNPs that had missing data (no genotype call) for either duplicate.

Linkage map creation, QTL mapping, and combined linkage disequilibrium analysis

Linkage maps were created for each of the four F_2 families independently, and phase information was estimated in OneMap following the Outcrossing Populations tutorial ('OneMap' version 2.1.3; Margarido, Souza, & Garcia, 2007). The package 'vcfR' was used to load the raw, filtered SNP file (.vcf) into R for each family before linkage map construction ('vcfR' version 1.9.0; Knaus & Grünwald, 2017). For each family, redundant markers and markers with segregation distortion ($\alpha < 0.05$ after Bonferroni correction) were removed before map building, and only markers present in 90% of the individuals were used. Markers were assigned to linkage groups according to chromosome information from the eastern oyster genome (10 chromosomes, Gómez-Chiarri *et al.* 2015). We thinned each linkage group to 50-100 markers to make mapping easier (i.e. less computation) and because an excessive number of markers are not needed given the architecture of the F_2 families (i.e. high linkage between markers). Markers were then ordered sequentially according to their location in the genome and phase information was generated using the 'map()' function (Margarido et al., 2007). A final linkage map was created for each family with linkage groups in correct chromosome order, and the OneMap file outputs were converted to R/qtl format using the OneMap-to-Rqtl-4waycross script that was written for this project (https://github.com/lexymccarty/OneMap-to-Rqtl-4waycross).

QTL mapping was performed in R/qtl for each family independently (version 1.44-9; Broman, Wu, Sen, & Churchill, 2003). Individuals with identical genotypes (>90% identical markers) were identified and one individual from each pair was omitted. Markers with identical genotypes (duplicate markers) and markers with segregation distortion (Chi-Square $P < .001$) were also removed. Conditional genotype probabilities were calculated ('calc.genoprob') for each family and a two-part single-QTL model (model='2part') was used for phenotype day to death for families 11, 22, and 43 since the phenotype spikes at day 50, representing individuals that survived the low salinity challenge (Broman, 2003). In this scenario, we first consider the binary trait where an individual with QTL genotype g has probability π_g of having the nonzero phenotype (mortality in the low salinity challenge). If the individual has the nonzero value (mortality), the value is assumed to be normally distributed with mean day to death (μ_g) and standard deviation (σ) (Broman, 2003). Therefore, we log-transformed the day to death phenotype to follow a normal distribution. All 2-part QTL models were run with 1000 permutations to determine the 5% significance threshold at the genome-wide level. For family 65, a one-dimensional genome scan was performed with a single-QTL model ('scanone' module) for day to death since all individuals died. Significant QTL were incorporated into a model ('fitqtl' module) to investigate the effect of each QTL on the two traits of interest, survival and day to death, for each family since effect models cannot be fit for 2-part single-QTL models. Finally, 'refineqtl' was used to refine the estimated location of QTLs and 'fitqtl' was performed on the refined locations to investigate model improvement.

Linkage disequilibrium analysis was performed on the filtered SNPs from a total of 372 individuals across the four families in TASSEL (version 5.2.57, Bradbury et al., 2007). The genotype table was filtered for sites with a minimum minor allele frequency (MAF) of 0.05,

maximum frequency of 1, and for sites present in at least 150 individuals (Bradbury et al., 2007). Population structure was analyzed using analysis of principle components (PCA; see Results 3.4 Figure 3). Within TASSEL, genotypes from the filtered table were converted to numbers, where the homozygous major genotype is coded as 1, homozygous minor is 0, and heterozygous is 0.5. Missing values were then imputed using Euclidean distance and the 5 nearest neighbors (Bradbury et al., 2007). Once all missing values were imputed, a PCA was conducted on the imputed genotype table (Price et al., 2006). A kinship matrix using Centered_IBS was calculated from the filtered genotype table to generate pairwise relatedness coefficients for each marker (K matrix) (Bradbury et al., 2007).

Combined linkage disequilibrium (LD) analysis was performed using a mixed linear model (MLM) in TASSEL with the four generated files: the phenotype file filtered for the trait of interest (day to death or survival), the combined filtered genotype table, the first 10 components of the PCA (from TASSEL, explaining 59% of the variation), and the kinship matrix (K) (Bradbury et al., 2007; Lu et al., 2010):

Phenotypic trait = Marker effect + PCA components + K + residual

Both the PCA and K matrix were used to minimize spurious associations (Lu et al., 2010). Two total MLMs were conducted, one for each of the two traits (day to death and survival). Significant thresholds were determined for each model using the Bonferroni correction: α/N, where α is the significance level of 0.05 and N is the total number of effective tests (determined using 'simpleM' method) to account for any linkage disequilibrium between SNPs (Benjamini and Hochberg, 1995; Gao et al., 2010, 2008). Manhattan plots were created for each trait using the 'qqman' package in R (version 0.1.4; Turner, 2014). All significant QTLs from the QTL mapping and combined LD analyses were located in the eastern oyster reference genome

C_virginica-3.0 (GenBank accession GCA_002022765.4; Gómez-Chiarri et al., 2015). Each gene, or gene closest to each significant SNP, was investigated for annotation and function using the NCBI Genome Data Viewer, and corresponding GO terms were queried for each gene/protein sequence (Johnson and Kelly, 2020). R version 3.6.1 was used for all necessary analyses (R Core Team 2020).

Genomic prediction and trait correlation

The filtered SNP file used in the combined LD analysis was used to estimate genomic prediction accuracies using Bayesian linear regressions in the statistical package BGLR (version 1.0.8; Pérez & De Los Campos, 2014). The genotype file was read into R using the BEDMatrix package (version 2.0.3; Grueneberg & de los Campos, 2019) and missing genotypes were imputed using *knncatimpute* in the 'scrime' package using the 4 closest neighbors (version 1.3.5; Schwender, 2012). Once imputed, genotypes were recoded into BGLR format (AA = 0, Aa = 1, aa = 2) and marker effects were estimated using Bayesian Ridge Regression (BRR) and the BayesB prior for both traits: survival was modeled as a binary trait and day to death was modeled as a censored trait with a minimum value of 0 and a maximum value of 36. The accuracy of marker selection was assessed by randomly splitting individuals into five testing (20%) and training (80%) sets for cross-validation, and phenotypes of the testing individuals were coded as missing in the training set. This process was repeated five times for each trait and each prior. Realized prediction accuracy was calculated as the correlation between the predicted marker values of the testing set and the actual phenotypes divided by the square root of the trait heritability when all phenotypic data is included.

Genomic estimated breeding values (GEBVs) for both traits were estimated in BGLR following the model below:

$$y_i = u + Z_i\beta_i + e_i$$

where y is the observed phenotype (either survival or day to death) of the individual, u is the average population phenotype, Z_i is the marker-derived matrix of genetic relatedness between individuals (GRM), β_i is the vector of SNP effects, and e_i is a vector of residual error. Both trait models were fit using a Bayesian Reproducing Kernel Hilbert Spaces Regression (RKHS), but survival was fit with a logit link function because it is a binary trait. Models were assessed by creating five random 20%/80% testing/training validation sets, which was repeated 10 times. Narrow-sense heritability (h^2) was estimated as the additive genetic variance from the GRM over the total phenotypic variance (including both the GRM and residual variance), as follows:

$$h^2 = \sigma^2_a / \sigma^2_p$$

where σ_a is the additive genetic variance from the GRM, and σ_p is the total phenotypic variance, which is the sum of the additive (σ^2_a) and residual (σ^2_e) variance. For survival, the residual variance is fixed at 1, so the heritability becomes:

$$h^2 = \sigma^2_a / (\sigma^2_a + 1)$$

Five independent models were run to estimate heritability for each trait, and the average of the five estimates is reported. Number of iterations, burn in, and thinning parameters were determined by assessing the convergence and autocorrelation for all models using the 'coda' package in R (version 0.19-3; Plummer et al., 2006; Villemereuil, 2012). All models were run for 2.5 million iterations with a thin of 1,000 after a burnin of 500,000 for both traits. For both day to death and survival, BayesB and RKHS models were rerun with the same parameters above, but after removing all SNPs within the significant region on chromosome 1 (21,800,000 – 32,800,000 base pairs), leaving a total of 27,273 SNPs. Realized accuracies were estimated using

the same validation scheme as above. To test the effect of SNP thinning on genomic selection prediction accuracy, we created randomly thinned datasets consisting of 25,000, 20,000, 15,000, 10,000, 5,000, 1,000, 500, 250, 100, 50, 25, 10, and 2 markers, with three random replicates of each thinned marker dataset. We estimated the realized prediction accuracy for each thinned dataset using a 20%/80% testing/training cross validation scheme as described above using RKHS and BayesB models. Thinned datasets of 25,000, 20,000, 15,000, and 5,000 were omitted for the BayesB models due to computational effort and because the accuracies did not decrease during these intervals. The genetic correlation between the two low salinity challenge traits, survival and days to death, was assessed using a bivariate animal model implemented in ASReml-R using the genotype-derived relationship matrix (Gilmour et al., 2015).

Results

Survival during acute low salinity challenge

The acute low salinity challenge (2.2) induced mortality in all four F_2 families over the 36-day challenge period. Ninety-two percent, 82%, 90%, and 100% of oysters died from family 22, 11, 43, and 65, respectively. Most of the mortality during the experiment (310 oysters, 80%) occurred from days 9 – 21 of the challenge, with peak mortality occurring on day 11 with 53 total dead oysters (Figure 1). Peak mortality occurred on day 11 for family 22 (14 oysters, 14%) and 65 (23 oysters, 23%), on day 14 for family 11 (16 oysters, 15%), and on day 17 for family 43 (11 oysters, 14%). All families had individuals remaining alive after the challenge except for family 65.

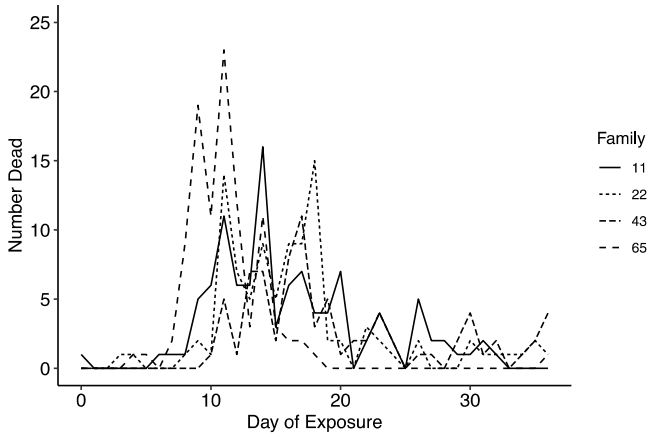


Figure 1. Number dead for each of the four F_2 families over the 36-day acute low salinity (2.2) challenge. N = 132, 111, 114, and 106 for families 11, 22, 43, and 65, respectively.

Sequencing results

All libraries yielded high quality read data, with > 93%, > 70%, and > 94% of raw reads retained after demultiplexing libraries 1, 2, and 3, respectively, which resulted in discovery of 4,092,824 SNPs for 489 individuals from the four F_2 families. The total number of SNPs was significantly reduced after filtering, and the majority of SNPs were removed when filtering for SNPs present in 90% of individuals, a minimum quality score of 30, minor allele count of 3, minor allele frequency of 0.05, and a minimum average depth of 20 reads. A total of 28,638 SNPs across 399 individuals remained after applying filters. Mean read depth per site, after accounting for the number of individuals in each group, was 73 and average missingness for each individual was 3.4%. Duplicated samples had an average genotype error rate of 1.97% across all individuals (all families) and within-family error rates ranged from 1.07% (family 22) to 2.74% (family 11).

Linkage map construction and QTL mapping

Final linkage maps were created from 123, 100, 91, and 95 individuals using a total of 380, 288, 370, and 400 genotyped markers (after thinning to 50 – 100 SNPs per chromosome), for family

11, 43, 22, and 65, respectively. A significant QTL on chromosome 1 was identified by the 2-part model (day to death conditional on survival) for family 11 and 43 (Figure 2). All significant markers were located between 21,000,000 and 26,000,000 base pairs on chromosome 1 (Table 1). For Family 11, a significant QTL for the 2-part model (day to death conditional on survival; red line, Figure 2) was located in the uncharacterized LOC111116948 gene on Chr1 in the eastern oyster genome. After incorporating this QTL into a single-QTL model for day to death and after refining the position, the QTL was located in the E3 ubiquitin-protein ligase UBR5-like gene (Table 1) and explained 10.4% of the model variation, but was not above the LOD significance threshold at the genome-wide level. When this QTL was incorporated into the single-QTL model for survival and after position refinement, the QTL was located in the uncharacterized LOC111128605 gene and explained 10.5% of the model variation, but was not above the significance threshold (Table 1). This QTL, before refining, was just below the LOD significance threshold for the probability of surviving from the 2-part model (0.5 below; black line, Figure 2).

For family 43, the QTL region on chromosome 1 was significant for day to death conditional on survival (red line), the probability of survival (black line), and mean day to death (grey line, Figure 2). This significant QTL was located in the nuclear receptor coactivator 2-like gene in the eastern oyster genome. After incorporation into the single-QTL model for day to death and following refinement, this QTL was above the LOD significance threshold and explained 50.55% of the single-QTL model variation (Table 1). After position refinement, the QTL was located in the solute carrier organic anion transporter family member 4A1-like gene (Table 1). When incorporated into the single-QTL model for survival and after position refinement, the QTL was still located in the nuclear receptor coactivator 2-like gene, explained

32.08% of the model variation, and was above the LOD significance threshold (Table 1). There were no QTL above the significance threshold for families 22 and 65.

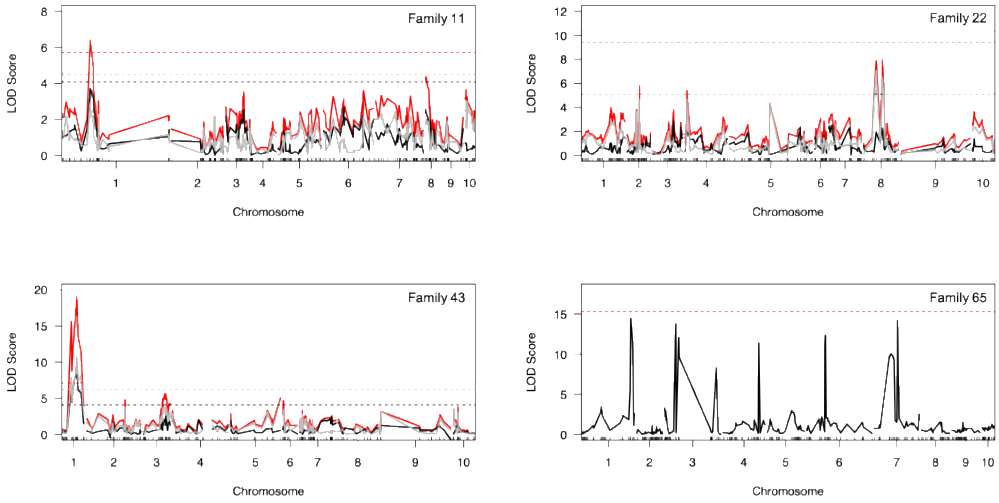


Figure 2. LOD plots for QTL identified from the 2-part model, day to death conditional on survival, for family 11 (top left), 43 (bottom left), and 22 (top right). LOD plot in the bottom right shows the QTL identified from the single-QTL scan for day to death for family 65. For the 2-part models, red lines indicate the QTL associated with mean day to death conditional on the probability of survival ($LOD_{p\mu}$), black lines represent QTL associated with the probability of survival (LOD_p), and grey lines indicate QTL associated with mean day to death (LOD_μ). Horizontal, dotted lines indicate the 5% significance threshold at the genome-wide level after 1000 permutations for each respective test (by color).

Table 1. Significant QTL (above the LOD threshold) identified from the 2-part scans incorporated into independent models ('fitqtl') for both day to death and survival for families 11 and 43. Significant markers were refined for position ('refineqtl') and then incorporated into each model to get percent variance (% Var) explained and a model significance value (p-value). Family 22 and 65 are excluded because there were no peaks above the LOD threshold.

Family	Trait	Chr	Position (bp)	% Var.	*P*-value (c^2)	LOD Score	Gene
11	Day	1	21875299	10.4	0.0136	2.316	E3 ubiquitin-protein ligase UBR5-like
	Survival	1	25149524	10.5	0.004	2.915	[1]uncharacterized LOC111128605
43	Day	1	21924061	50.55	< 0.001	9.32*	solute carrier organic anion transporter family member 4A1-like
	Survival	1	25873768	32.08	< 0.001	7.979*	nuclear receptor coactivator 2-like

* above LOD threshold determined by single-QTL models ('scanone')
[1] gene located closest to the significant QTL

Combined LD analyses

For the MLMs, the first 10 PCAs (explaining 59% of the variation) were incorporated to account for population structure. When looking at the scree plot of variance explained for each principal component, there was a severe drop after PCA component 3 (Figure 3 A, B). Four distinct populations clustered when plotting PCA components, which represents the four F_2 families. The population structure completely disappeared when plotting component 6 and 7 (Figure 3 C, D). The scree plot suggests incorporating the first 3 components, while the clustering approach suggests incorporating the first 4. Therefore, we decided to conservatively incorporate the first 10 PCAs, accounting for ~59% of the variation, into the GLMs to account for population structure in our samples.

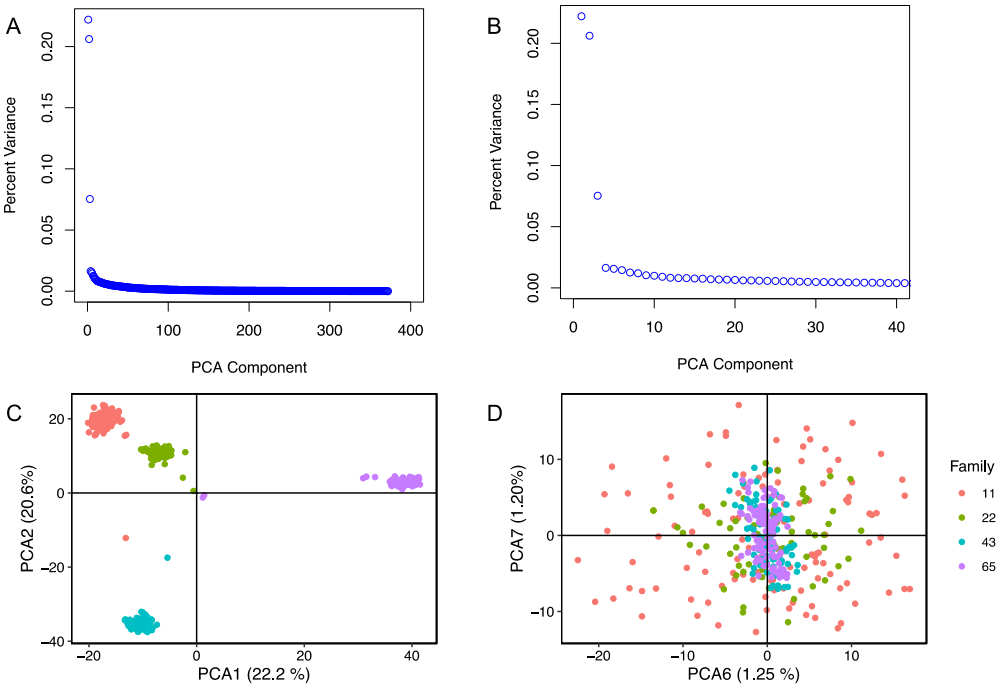


Figure 3. Scree plot showing the percent variance explained by A) all 372 PCA components, and B) the first 40 components. PCA plots showing C) population structure when plotting the first two components against each other (k = 4), and D) the lack of structure when components 6 and 7 are plotted.

The combined linkage disequilibrium analysis on a total of 28,502 SNPs identified regions on chromosome 1 and 7 significantly associated with both survival and day to death. There were a total of 87 and 46 SNPs for survival and day to death, respectively, that were above the significance threshold of 1.95×10^{-6} after correcting for the number of effective tests (0.05/25,685 effective tests; Table S1; Gao et al., 2010, 2008; Yoav & Hochberg, 1995). The same 41 SNPs were significant for both day to death and survival, and an additional 5 and 46 SNPs were exclusively significant for day to death and survival, respectively (Supplemental

Table 1). Models for both traits revealed a significant peak on chromosome 1 from 21,800,000 – 28,600,000 base pairs, as well as a significant SNP on chromosome 7 at base pair 7,251,580 (significance threshold = 5.7, Figure 4A, C).

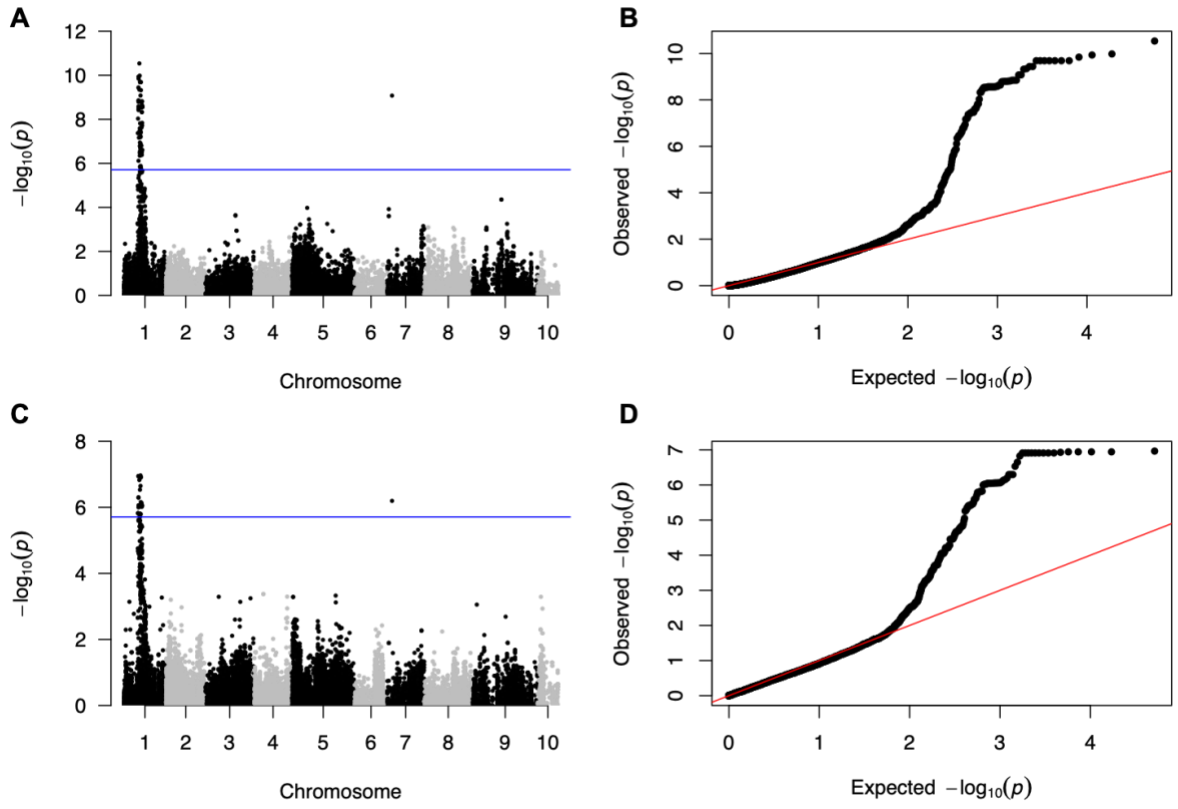


Figure 4. Combined linkage disequilibrium analysis of survival (A, B) and day to death (1-36 days, C, D) for the four recombinant families exposed to acute low salinity (2.2) for 36 days. QQ plots (right) and Manhattan plots (left) depicting -$\log_{10}(p)$ values from the combined linkage disequilibrium analysis for genome-wide SNPs and survival (A) and day to death (B). Blue horizontal lines in Manhattan plots represent significance threshold after correcting for multiple tests.

Comparing between traits (survival and day to death), significant SNPs were located in a total of 16 characterized genes, seven of which were shared between the two traits (Table 2). For the survival MLM, the most significant SNP (chromosome 1, base pair 23,957,309) was not located in a gene, but the next most significant SNP was located in the ATP-dependent 6-phosphofructokinase-like gene with an R^2 value of 0.128 (Supplemental Table 1, Table 2 bold). The most significant SNP (chromosome 1, base pair 25,724,354) from the day to death MLM was also located outside of a gene, but the next most significant SNP was located in the metalloproteinase inhibitor 3-like gene and had an R^2 value of 0.145 (Supplemental Table 1, Table 2 underline). Including all SNPs within the significant QTL peak on chromosome 1 and the single significant SNP on chromosome 7 accounted for 8.97 and 6.51 of the total model variation (R^2) for survival and day to death, respectively (Table 2). When grouping the 16 identified genes by their predicted function, 31% (5 genes) had functions related to DNA/RNA function and repair: coiled-coil domain-containing protein 13-like, E3 ubiquitin-protein ligase UBR5-like, nuclear receptor coactivator 2-like, nucleolar MIF4G domain-containing protein 1-like, and rho GTPase-activating protein 190-like). Another 44% (7 genes) had functions related to ion binding and membrane transport: cadherin-23-like, gamma-aminobutyric acid type B receptor subunit 2-like, metalloproteinase inhibitor 3-like, monocarboxylate transporter 14-like, solute carrier organic anion transporter family member 4A1-like, transient receptor potential cation channel subfamily M member 1-like, and zinc transporter 2-like. The remaining 25% (4 genes) had other predicted functions, such as lipid synthesis and transport and response to oxidative stress: ATP-dependent 6-phosphofructokinase-like, oxidation resistance protein 1-like, extended synaptotagmin-2-like, choline/ethanolaminephosphotransferase 1-like (Table 2).

Table 2. Genes with significant SNPs from the Combined Linkage Disequilibrium analysis for both survival and day to death in extreme low salinity. Chromosome of annotated gene within the genome is included, along with the significant number of SNPs detected within that gene and their total R^2. If the gene was present in both analyses, values for survival and day to death are separated by "|". Gene where most significant SNP was located is bolded and underlined for survival and day to death, respectively. Gene function is indicated with either D (DNA), T (Transport), or O (other).

Trait	Annotated Gene	Chr	# of SNPs	R2	Function
Both	E3 ubiquitin-protein ligase UBR5-like	1	2 | 2	0.171 | 0.234	D
	metalloproteinase inhibitor 3-like	1	5 | 4	0.583 | 0.584	T
	monocarboxylate transporter 14-like	1	3 | 2	0.350 | 0.275	T
	nuclear receptor coactivator 2-like	1	8 | 5	0.856 | 0.715	D
	nucleolar MIF4G domain-containing protein 1-like	7	1 | 1	0.113 | 0.133	D
	oxidation resistance protein 1-like	1	3 | 1	0.295 | 0.142	O
	rho GTPase-activating protein 190-like	1	2 | 2	0.197 | 0.247	D
	not in a gene	1	25 | 20	2.76 | 2.96	
	uncharacterized gene	1	18 | 7	1.67 | 0.974	
Survival	**ATP-dependent 6-phosphofructokinase-like**	1	1	0.128	O
	cadherin-23-like	1	2	0.184	T
	choline/ethanolaminephosphotransferase 1-like	1	4	0.388	O
	coiled-coil domain-containing protein 13-like	1	7	0.709	D
	extended synaptotagmin-2-like	1	2	0.172	O
	gamma-aminobutyric acid type B receptor subunit 2-like	1	1	0.087	T
	solute carrier organic anion transporter family member 4A1-like	1	1	0.106	T
	zinc transporter 2-like	1	2	0.205	T
Day to Death					
	transient receptor potential cation channel subfamily M member 1-like	1	2	0.247	T
	Totals:		**87 | 46**	**8.97 | 6.51**	

Genomic prediction, heritability, and trait correlation

Realized prediction accuracies including all SNPs ranged from 0.489 – 0.547 and 0.507 – 0.57 for day to death and survival, respectively (Figure 5). Realized accuracies for both traits were highest for the marker models with the BayesB prior, followed by BRR and RKHS (Table 3). For both traits, removing significant SNPs on chromosome 1 resulted in only a small reduction in accuracy values for both RKHS and BayesB, and the reduction was largest for both traits using BayesB. After removing SNPs on chromosome 1, accuracies for survival decreased

by 0.056 and 0.1 for RKHS and BayesB, respectively, and accuracies for day to death decreased by 0.029 and 0.12 for RKHS and BayesB, respectively (Figure 5 and Table 3).

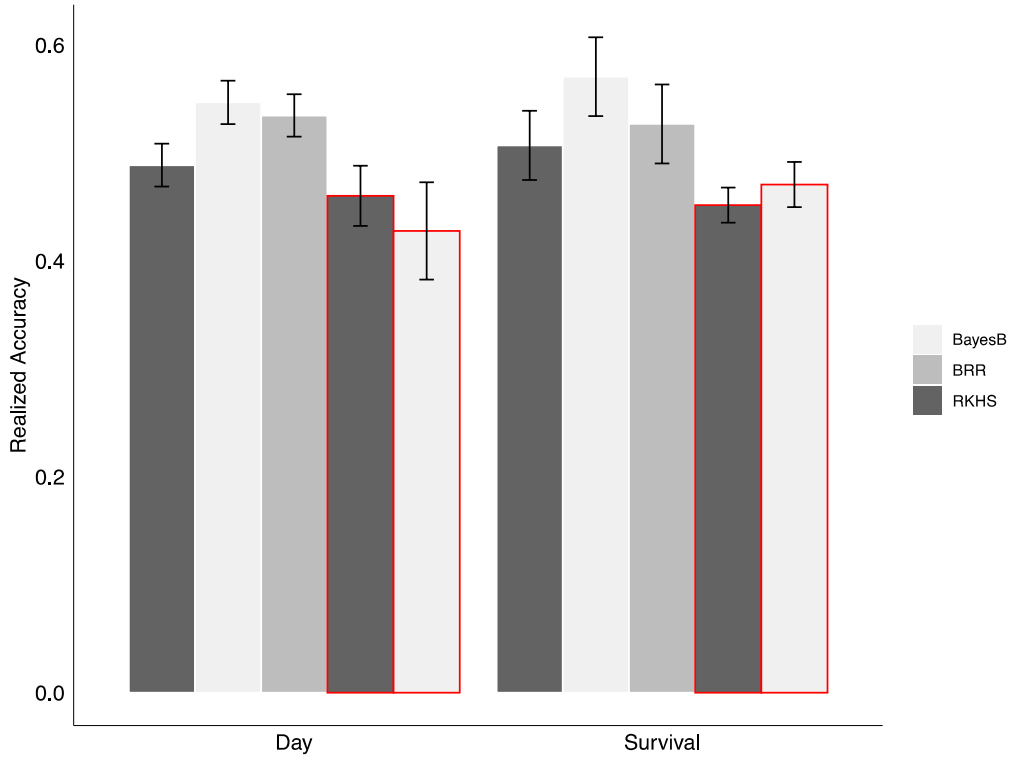


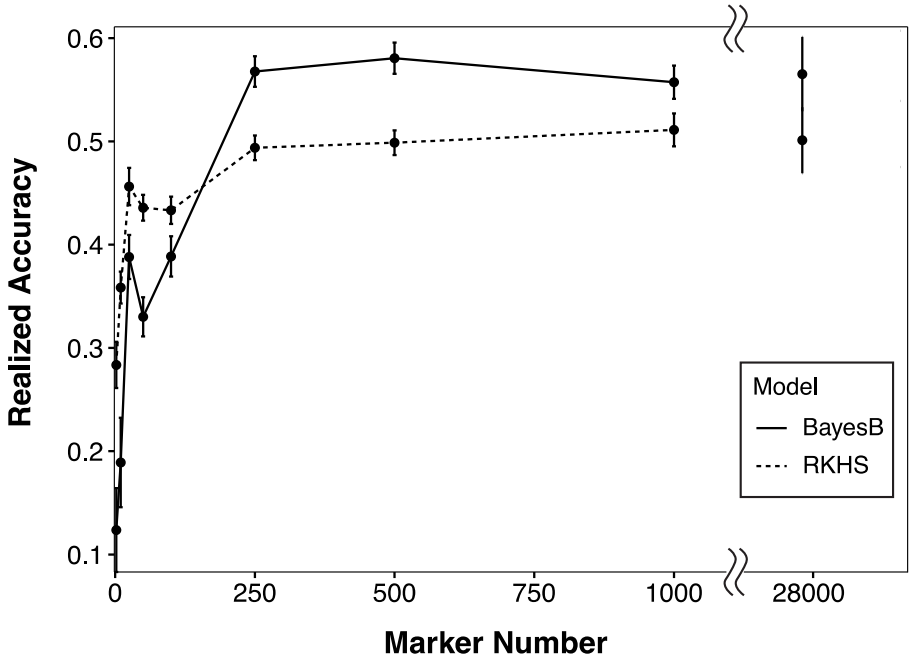
Figure 5. Realized genomic prediction accuracies for survival and day to death in the extreme low salinity challenge. Regression models were run for both traits including all SNPs and after removing SNPs in the significant region on chromosome 1 (red outline). Each bar represents the average value of the 50 and 25 separate 20%/80% cross-validation sets for RKHS and marker models (BayesB, BRR), respectively, divided by the square root of the respective estimated heritability value, 0.406 for day to death and 0.595 for survival. Error bars represent standard error of the mean.

Table 3. Realized accuracy estimates (± SEM) for survival and day to death in the acute low salinity challenge. Accuracies were estimated using a 20%/80% testing/training validation set for all regression models (RKHS, BRR, or BayesB) using all SNPs (All) and after removing SNPs in the significant region on chromosome 1 (No Chr1).

Trait	Markers	Model	*Realized Accuracy (± SE)
Survival	All	RKHS	0.507 (0.032)
		BRR	0.527 (0.037)
		BayesB	0.571 (0.036)
	No Chr1	RKHS	0.451 (0.016)
		BayesB	0.471 (0.021)
Day to death	All	RKHS	0.489 (0.020)
		BRR	0.535 (0.020)
		BayesB	0.547 (0.020)
	No Chr1	RKHS	0.460 (0.028)
		BayesB	0.428 (0.045)

* accuracy / $\sqrt{h^2}$; h^2 survival = 0.539; h^2 day to death = 0.406

Realized prediction accuracies decreased when the number of SNP markers used was reduced (thinned) to below 250. For example, realized accuracy dropped to 0.43 and 0.39 for RKHS and BayesB, respectively, when models were run with 100 markers (Supplemental Figure 1).



Supplemental Figure 1. Average realized genomic selection accuracies for BayesB and RKHS using reduced marker number (thinned) datasets. Each point represents the average accuracy for ten 20%/80% testing/training cross validation for three different thinned data sets with a marker number of 25000, 20000, 15000, 10000, 5000, 2000, 1000, 500, 250, 100, 50, 25, 10 and 2 markers. Error bars represent the SEM. Note that the X-axis is broken between 1000 and 28000 markers.

Narrow-sense heritability estimates were moderate to high for both traits. The heritability estimate for day to death was 0.406 (Confidence interval: 0.231 – 0.595), which is slightly lower than the estimate for survival, 0.539 (CI: 0.326 – 0.750). The genetic correlation between the two low salinity challenge traits, survival and day to death, was large and significant, 0.867 ± 0.027.

Discussion

An experimental challenge at extreme low salinity (salinity < 3) was conducted with four F_2 eastern oyster families to examine the genetic basis of extreme low salinity survival. QTL mapping and combined LD analysis using genome-wide SNPs revealed significant QTL on chromosome 1 and 7 for both traits, survival and day to death. Genes within, or proximal to, identified QTN had functions related to DNA/RNA function and repair, ion binding and membrane transport, and in the response to stress. Genomic prediction accuracies (0.48 – 0.57) suggest that genomic selection is a viable option for improving survival in acute low salinity for the eastern oyster, at least based on this dataset. However, future studies with a more appropriate experimental design are necessary. Furthermore, a larger genomic prediction accuracy of the BayesB regression model, along with the lack of substantial decrease in prediction accuracy when removing SNPs within the significant QTL region on chromosome 1, suggest that survival

in extreme low salinity may be controlled by many genes of small and potentially unequal effect, as opposed to being controlled by relatively few major-effect QTL.

QTL and combined disequilibrium analyses

QTL mapping and combined LD analysis revealed similar QTL on chromosome 1 related to both survival and day to death. The four significant QTL (Table 1) were located within the significant peak on chromosome 1, from 21,800,000 – 28,600,000 base pairs, detected by the combined LD analysis. For the combined LD analyses, the SNPs located within the significant QTL on chromosome 1 (87 SNPs for survival and 46 SNPs for day to death) explained a total of 8.97% and 6.51% of the total phenotypic variation for survival and day to death, respectively, with the most significant SNP explaining < 0.1% for both traits. For the QTL mapping, a significant QTL explaining a large portion of the total variation (32% for survival and 50% for day to death) was detected for only one of the families (43) after refinement, and the insignificant QTL (after refinement) detected in family 11 explained 10% of the total variation for both traits. The relatively small contribution of our detected (major) QTL from both analyses suggests that many other markers of relatively small effect will likely have a combined large effect on the phenotype. Thus, survival in extreme low salinity (< 3) may be controlled by additional genes not identified in this analysis. Aside from one study (Sauvage et al., 2010), most QTL studies in bivalve shellfish have examined 2 or fewer families (Fang et al., 2021; Guo et al., 2012; Wang et al., 2016; Zhan et al., 2009; Zhong et al., 2014). QTLs of similar magnitude (8 – 40 percent variance explained) identified for multiple traits in salmonids have been proposed for incorporation into MAS programs (Ayllon et al., 2015; Barson et al., 2015; Boison et al., 2019; Gonen et al., 2015), but these QTL were validated across multiple populations. In this study, the significant QTL identified on chromosome 1 was detected in only two families, which happen to

be the two largest families analyzed, indicating that these families could be driving the detection of the QTL in our combined LD analyses. Thus, the QTL require further validation across other families and populations to determine if they are generally useful and associated with the trait, or if they are specific to the genetic background of the F_2 families tested. Our sample size was relatively small (< 400) and further analyses should be conducted on a larger sample size with individuals from many populations. A sample size larger than 1000 is recommended for higher resolution when detecting QTL (Barría et al., 2018; Houston et al., 2020), and the possibility of conducting a GWAS of this magnitude is becoming more practical as genomic tools for the eastern oyster continue to be developed (i.e. SNP array; Houston et al., 2020; Thongda et al., 2018).

QTL mapping and combined LD analysis results suggest that survival and day to death are genetically similar traits. The similarity in the three LOD score curves for the QTL mapping models (2-part: day to death conditional on survival, survival as binary, and day to death with a normal distribution) suggest that similar QTL were identified when analyzing either trait independently. Similarly, the same major QTL region on chromosome 1 was also detected in the combined LD analysis for both traits, and 85 significant SNPs were shared within the same 6.8 million base pair region (21,800,000 – 28,600,000 base pairs). A high genetic correlation was detected between the two traits (0.867 ± 0.027), and large genetic correlations (0.95) have previously been detected for disease-related survival traits (survival and day to death) in both salmon and trout (Barría et al., 2018, 2020; Bassini et al., 2019; Palti et al., 2015). The finding that survival and day to death are genetically similar suggests that either trait could be used in future assessment of extreme low salinity survival.

Combined LD analysis provided increased resolution and statistical power because all individuals were analyzed together (larger sample size). Combined LD analysis was able to detect not only the significant region on chromosome 1, but also an additional significant region on chromosome 7. Suggestive peaks for the combined LD analysis were present just below the significance threshold on chromosomes 5, 7, 8, and 9, and may contribute to the overall variation in this trait (Figure 4). In contrast, the family-specific QTL analyses did not detect these additional QTL, but there does seem to be a suggestive QTL on chromosome 8 for family 22 (Figure 2). Previous studies have also observed increases in detection ability for combined LD mapping (Lu et al., 2010; Xiong and Jin, 2000). In our scenario, the combined LD analysis provided the most powerful analysis, but is complemented by the independent QTL mapping results.

Functional analysis of QTL and SNPs

For both survival and day to death, the four major QTL and 133 significant SNPs were located within, or proximal to, a total of 16 annotated genes. These genes have functions belonging to three major categories: DNA/RNA function and repair, ion binding and membrane transport, and the response to stress. Our results build upon previous transcriptomic studies of oysters and highlight potential genes and physiological processes underlying survival in extreme low salinity (< 3).

Five of the 16 QTL-associated genes were annotated with functions related to DNA/RNA function and repair. Four of these genes (E3 ubiquitin-protein ligase UBR5-like, nuclear receptor coactivator 2-like, nucleolar MIF4G domain-containing protein 1-like, and rho GTPase-activating protein 190-like) have functions related to RNA binding and gene transcription. Previous work examining the transcriptomic response of eastern oysters to a salinity of 8 and

Olympia oysters to a salinity of 5, revealed the strongest enrichment for genes related to DNA replication and transcription (Eierman and Hare, 2014; Maynard et al., 2018). The enrichment or detection of genes involved in gene transcription at low salinity might reflect the importance, and necessity, of increasing DNA function and transcription of genes responsible for conformation to stressful low salinities. For example, in eastern oysters, the rho GTPase-activating protein 190-like gene was previously found to be enriched at low salinity (salinity 8) and is considered an important osmoregulatory candidate (Eierman and Hare, 2014). Rho proteins are also involved in anti-apoptotic processes (reviewed in Li *et al.* 2015), and infection of Pacific oyster hemocytes with vectors expressing the California sea hare (*Aplysia californica) rho* gene reduced β-adrenoceptor-induced apoptosis (Lacoste et al., 2002). Upregulation and expression of many antiapoptotic genes and pathways is a known stress response in oysters (Zhang et al., 2016), and rho GTPase-activating protein 190-like may play an important role in preventing apoptosis to maintain internal homeostasis and cell integrity during extreme low salinity exposure.

The majority of genes (7/16) proximal to or underlying QTL were related to membrane transport and ion binding. Oysters are osmoconformers that regulate the concentration of inorganic ions (Na^+, Ca^{2+}, and Mg^{2+}) and free amino acids within their cellular fluid to maintain osmotic balance and conform to the salinity of their surrounding environment (Pierce, 1982, 1971b; Shumway, 1977b, 1977a). SNPs significantly associated with variation in low salinity survival were detected in cadherin-23-like and transient receptor potential cation channel subfamily M member 1-like, both of which are transmembrane proteins that play a role in calcium ion binding and cation channel activity (Mège and Ishiyama, 2017; Venkatachalam and Montell, 2007). Induction of calcium dependent pathways is a documented response to salinity stress in bivalves (Eierman and Hare, 2014; Gong et al., 2021; Shumway, 1977b; Zhang et al.,

2016), thereby regulating calcium metabolism, transport, and internal fluid osmolality. Additionally, expression of transient receptor proteins are known to be indicative of stress (Venkatachalam and Montell, 2007), and are specifically involved in the thermal stress response in both the Pacific and Portuguese oyster (Fu et al., 2021). Zinc transporter 2-like gene has functions specifically related to zinc ion binding, and this gene was previously shown to be associated with osmoregulation in the eastern oyster (Eierman and Hare, 2014). A significant SNP was also detected in the metalloproteinase inhibitor 3-like gene, which prevents the breakdown of metalloproteins. Metalloenzymes, which are superoxide dismutases with a bonded metal (Cu/Zn or Mn), are part of the defense system against oxidative stress (Park et al., 2009; Rudneva, 1999). These two genes suggest that zinc ion binding plays an important role in the response to extreme low salinity, and a moderate heritability for zinc ion accumulation in Fujian oysters (Wu et al., 2019) could point to a specific mechanism responsible for the observed variation in survival.

The remaining four genes identified from the genome-wide analyses had functions related to oxidative stress and protein regulation. Oxidative stress results from an excess of free radicals in an organism's cells in response to an environmental stressor (Lushchak, 2011; reviewed in Rivera-Ingraham & Lignot, 2017), and expression of antioxidative genes are commonly used to indicate oyster health and stress (Zhang et al., 2016). Therefore, the significant SNPs detected in the oxidation resistance protein 1-like gene are not surprising and suggest that extreme low salinity tolerance could be influenced by genetic variation in oxidative response pathways. Additionally, maintenance of ion gradients during osmoregulation is one of the most ATP-demanding processes (Hand and Hardewig, 1996; Sokolova et al., 2012). Many genes involved in protein regulation were previously found to be significantly upregulated in the

Pacific oyster after challenge to a low salinity of 8 (Wang et al., 2012). The identification of SNPs associated with variation in low salinity tolerance within the ATP-dependent 6-phosphofructokinase-like gene support the notion that glycolysis and energy metabolism are likely important in maintaining cell function during salinity stress.

Genomic selection and heritability

Genomic selection prediction accuracies for all models ranged from 0.48 – 0.57 for both traits, which are slightly lower than ranges reported for production and disease-related traits in other bivalve species. Genomic selection prediction accuracies from GBLUP models for growth-related traits ranged from 0.54 – 0.67 and from 0.678 – 0.758 for resistance to Ostreid herpesvirus (OsHV-1-μvar) in the Pacific oyster (Gutierrez et al., 2020, 2018b). Other prediction accuracies were reported for growth related-traits in the Zhikong scallop (0.63 – 0.7) (Y. Wang et al., 2018) and for morphometric and edibility traits in the Portuguese oyster (0.4 – 0.79) (Vu et al., 2021). To our knowledge, there are no reported genomic prediction accuracies for environmental stress-related traits in aquaculture species for comparison, but studies of survival and day to death phenotypes for disease-related traits in finfish species have reported prediction accuracies as low as 0.21 (reviewed in Houston et al., 2020). Genomic prediction accuracies are affected by the underlying trait architecture, linkage disequilibrium structure, relatedness between training and testing sets, marker density, trait heritability, and sample size (Daetwyler et al., 2010; Dou et al., 2016; Habier et al., 2007; Meuwissen et al., 2001; Neves et al., 2012; Palaiokostas et al., 2019; Shengqiang et al., 2009). Therefore, the lower range of the prediction accuracies estimated here may be reflective of the underlying trait architecture.

More likely, the slightly lower range of genomic prediction accuracies may be an artifact of the small sample size (372 individuals) used and the relatedness between training and testing

sets (only 4 full-sibling families). The prediction accuracies estimated for low salinity survival in this study are most similar to those reported for growth-related traits in Yesso scallops (GBLUP, BayesB, RRBLUP: 0.3 – 0.6; Dou et al., 2016), where the authors assessed a population size of 349 scallops from 5 full-sibling families. Larger prediction accuracies were found for the Pacific oyster, Zhikong scallop, and Portuguese oyster where more families and larger sample sizes were utilized, e.g. greater than 500 individuals from at least 23 full or half-sibling families (Gutierrez et al., 2020, 2018b; Vu et al., 2021; Yangfan Wang et al., 2018). Caution should be taken when comparing our results to these larger, more comprehensive studies, as our experimental design and F_2 breeding structure represent fewer families/populations than are typically analyzed in GS studies. Future experimentation with a larger sample size and more populations may increase genomic prediction accuracies, as previous studies have found training population size to have a large effect on prediction accuracies (Ehret et al., 2015; Yangfan Wang et al., 2018). Nevertheless, the genomic prediction accuracies estimated here are on par with those in other marine bivalve studies, and larger than some previously reported accuracies for traits in finfish and shrimp species (reviewed in Houston et al., 2020).

Substantial thinning (reduction) in marker number (100 markers) was required to observe a noticeable reduction in prediction accuracy (0.07 and 0.18 decrease from the full marker model for RKHS and BayesB models, respectively). This result is likely a consequence of the F_2 breeding design employed. However, previous studies of marine animals utilizing more families and a more appropriate breeding design (i.e. > 20 half or full-sibling families) have also reported rather subtle decreases in prediction accuracy (~ 0.1) when sampling down to hundreds of markers (Gutierrez et al., 2020, 2018b). Overall, these results suggests that a relatively small number of markers (100s to a few thousand) may provide adequate genomic prediction

accuracies in experimental marine populations utilizing a family-based design. However, future work with larger sample sizes and a more appropriate breeding design is needed before making any major conclusions.

For both traits, accuracies were highest for regression models with the BayesB prior, followed by BRR and RKHS regression models. Accuracies differed by 0.064 and 0.058 between BayesB and RKHS models for survival and day to death, respectively, and we suspect that these differences arise from the weighting of the markers. For RKHS models, a traditional animal model replaced by a kernel matrix is executed, which is a matrix of genetic signal (or similarity) between individuals approximated from genetic effects (marker genotypes), as opposed to a traditional GBLUP where the genetic signal is equal to the marker genotypes (Morota and Gianola, 2014; Pérez and de los Campos, 2014). In RKHS models, one variance is shared and divided between all markers, so each marker is weighted the same and predicted to have the same minimal effect (Meuwissen et al., 2001). In Bayesian Ridge Regression (BRR), each marker has its own variance, but all are shrunk by the same shrinking parameter (Pérez and de los Campos, 2014). Lastly, the BayesB prior allows for variable selection, specifically size-of-effect shrinkage, where some markers have a small effect while the rest have minimal to no effect (Habier et al., 2011; Meuwissen et al., 2001; Morota and Gianola, 2014). The slight superiority in model performance by the BayesB prior could reflect the underlying nature of the trait, where survival in extreme low salinity is controlled by a few markers of small effect, such as those on chromosome 1 and 7, plus additional markers of minimal effect. Similarly, our genomic prediction accuracies decreased only slightly (0.029 – 0.12) when removing the significant region on chromosome 1, further supporting the notion that survival in extreme low salinity is polygenic in nature, and that regions other than those identified on chromosome 1

contribute to the overall trait variation. It is also worth investigating additional approaches (i.e. GBLUP, BayesA, BayesC) to ensure we have the model that best reflects the distribution of marker effects on our trait. While a Bayesian model (e.g. BayesB, BBR, BayesA, BayesC) may fit our trait best, the animal models (GBLUP and RHKS) are easier to implement with faster run times, and differences between GBLUP and Bayesian approaches have proven to be unsubstantial (Houston et al., 2020; Zenger et al., 2019).

The narrow-sense heritability for both survival ($h^2 = 0.539$) and day to death ($h^2 = 0.406$) were very similar to previously reported values using a pedigree-derived relationship matrix between half-sibling families ($h^2 \cong 0.4$, McCarty et al., 2020). Notably, the heritability estimate for survival was ~0.133 larger than the heritability estimate for day to death. Previous disease-resistance studies of salmon, red tilapia, and Nile tilapia all reported higher heritability estimates for threshold traits compared to their linear model counterpart (Shoemaker et al., 2017; Sukhavachana et al., 2019; Yáñez et al., 2013), which was suggested to result from a better fit of the threshold animal model for the binary trait (Barría et al., 2018). Moreover, higher narrow sense heritability values for disease-resistance traits in both Coho salmon and Nile tilapia corresponded to higher genomic prediction accuracies for these species (Barría et al., 2020, 2018), while lower heritability of disease resistance traits in the Portuguese oyster (0.1 – 0.11) resulted in lower prediction accuracies (0.24 – 0.3) (Vu et al., 2021). The correlation between high heritability values and high genomic prediction accuracies could provide an explanation for the higher genomic prediction accuracies for all survival models in this study.

Conclusion

Overall, this initial genome-wide analysis indicates that the genetic architecture of survival in low salinity for eastern oysters may be polygenic in nature, with significant QTL located on eastern oyster chromosome 1 and 7. Moreover, genomic selection (GS) appears to be a viable option for improvement of this trait in eastern oysters, which is encouraging as the implementation of GS continues to become more feasible for many aquaculture species. These preliminary results require further validation using larger sample sizes and the inclusion of more families or populations to corroborate detected QTL. Future GWAS experiments will help to elucidate the genomic architecture and the genes underlying low salinity tolerance in oysters, and will ultimately provide more information about the performance of genomic selection for improving this critical trait in oysters.

Data availability

All raw sequence data is deposited in NCBIs SRA (https://www.ncbi.nlm.nih.gov/sra) under project name (PRJNA756884). Phenotypic information for all individuals, raw and curated markers (SNPs) used in the combined LD analyses and genomic selection models, and genotype files used in Onemap and R/qtl are available in Figshare (https://doi.org/10.6084/m9.figshare.c.5577813.v1).

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Competing Interests

None of the authors have any competing interests.

Supplemental Table 1

Table S1. All significant SNPs from the combined linkage disequilibrium analysis for survival and day to death. SNPs are listed in consecutive order based on position (base pair) for each trait. SNPs not located in a gene are labeled NA.

Trait	Chromosome	Position (bp)	$-\log_{10}(p)$	R^2	Gene
Survival	1	21875334	7.37	0.085	E3 ubiquitin-protein ligase UBR5-like
	1	21875338	7.37	0.085	E3 ubiquitin-protein ligase UBR5-like
	1	21924061	8.03	0.106	solute carrier organic anion transporter family member 4A1-like
	1	22609492	8.42	0.101	metalloproteinase inhibitor 3-like
	1	22609495	8.48	0.134	metalloproteinase inhibitor 3-like
	1	22609519	9.44	0.116	metalloproteinase inhibitor 3-like
	1	22609547	9.44	0.116	metalloproteinase inhibitor 3-like
	1	22609572	8.58	0.116	metalloproteinase inhibitor 3-like

1	22609853	9.94	0.123	NA
1	22609860	6.11	0.082	NA
1	22609870	8.85	0.107	NA
1	22609871	8.79	0.139	NA
1	22609873	8.85	0.107	NA
1	22609877	8.85	0.107	NA
1	22771137	8.8	0.113	monocarboxylate transporter 14-like
1	22771138	9.84	0.128	monocarboxylate transporter 14-like
1	22771257	8.59	0.109	monocarboxylate transporter 14-like
1	23273232	7.17	0.107	NA
1	23956678	9.35	0.112	NA
1	23957309	10.54	0.128	NA
1	24490391	9.98	0.128	ATP-dependent 6-phosphofructokinase-like
1	24617297	7.18	0.094	oxidation resistance protein 1-like
1	24617410	6.35	0.1	oxidation resistance protein 1-like
1	24617505	7.59	0.1	oxidation resistance protein 1-like
1	24868758	6.55	0.086	uncharacterized
1	24868951	6.94	0.091	uncharacterized
1	24868958	6.47	0.085	uncharacterized
1	24868965	7.47	0.098	uncharacterized
1	24868981	7.47	0.098	uncharacterized
1	24868985	7.47	0.098	uncharacterized
1	24868987	7.47	0.098	uncharacterized
1	24880905	5.85	0.103	NA
1	24881232	6.47	0.096	uncharacterized
1	24881298	6.79	0.097	uncharacterized
1	24881322	6.79	0.097	uncharacterized
1	24899890	7.43	0.098	NA
1	25149428	9.69	0.117	NA
1	25149465	9.69	0.117	NA
1	25149558	9.69	0.117	NA
1	25149636	7.82	0.103	NA
1	25432258	6.69	0.088	extended synaptotagmin-2-like
1	25432290	6.45	0.084	extended synaptotagmin-2-like
1	25817658	8.54	0.123	NA
1	25817659	8.54	0.123	NA
1	25817660	8.54	0.123	NA
1	25817661	8.54	0.123	NA
1	25822728	9.69	0.117	nuclear receptor coactivator 2-like
1	25822843	5.86	0.076	nuclear receptor coactivator 2-like

1	25822852	9.69	0.117	nuclear receptor coactivator 2-like
1	25822872	8.81	0.117	nuclear receptor coactivator 2-like
1	25822888	9.69	0.117	nuclear receptor coactivator 2-like
1	25827663	9.08	0.11	nuclear receptor coactivator 2-like
1	25875693	7.23	0.101	nuclear receptor coactivator 2-like
1	25875726	7.2	0.101	nuclear receptor coactivator 2-like
1	25984434	6.9	0.1	uncharacterized
1	25995397	5.88	0.077	NA
1	25995483	6.37	0.083	NA
1	26221696	7.43	0.099	rho GTPase-activating protein 190-like
1	26229830	7.39	0.097	rho GTPase-activating protein 190-like
1	26272256	5.78	0.079	uncharacterized
1	26272261	5.78	0.079	uncharacterized
1	26458728	9.69	0.117	NA
1	26458755	9.32	0.112	NA
1	27131959	7.62	0.092	cadherin-23-like
1	27131971	7.62	0.092	cadherin-23-like
1	27205754	6.8	0.089	uncharacterized
1	27225046	8.78	0.105	NA
1	27877656	6.38	0.087	gamma-aminobutyric acid type B receptor subunit 2-like
1	27937253	8.32	0.098	uncharacterized
1	27937374	6.5	0.074	uncharacterized
1	27937383	8.32	0.098	uncharacterized
1	27937414	8.8	0.105	uncharacterized
1	28089010	8.64	0.102	zinc transporter 2-like
1	28089011	8.64	0.102	zinc transporter 2-like
1	28234691	8.82	0.105	NA
1	28342183	6.61	0.089	choline/ethanolaminephosphotransferase 1-like
1	28342185	6.61	0.089	choline/ethanolaminephosphotransferase 1-like
1	28342194	7.84	0.106	choline/ethanolaminephosphotransferase 1-like
1	28342449	7.72	0.104	choline/ethanolaminephosphotransferase 1-like
1	28589428	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589449	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589450	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589465	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589466	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589471	8.56	0.101	coiled-coil domain-containing protein 13-like
1	28589475	8.56	0.101	coiled-coil domain-containing protein 13-like
7	7251580	9.08	0.113	nucleolar MIF4G domain-containing protein 1-like

Day to death	1	21875334	5.82	0.117	E3 ubiquitin-protein ligase UBR5-like
	1	21875338	5.82	0.117	E3 ubiquitin-protein ligase UBR5-like
	1	22609492	6.95	0.147	metalloproteinase inhibitor 3-like
	1	22609495	6	0.175	metalloproteinase inhibitor 3-like
	1	22609519	6.3	0.131	metalloproteinase inhibitor 3-like
	1	22609547	6.3	0.131	metalloproteinase inhibitor 3-like
	1	22609853	6.3	0.131	NA
	1	22609870	6.95	0.147	NA
	1	22609873	6.95	0.147	NA
	1	22609877	6.95	0.147	NA
	1	22771138	6.06	0.137	monocarboxylate transporter 14-like
	1	22771257	6.06	0.137	monocarboxylate transporter 14-like
	1	23273232	5.79	0.148	NA
	1	23956678	6.93	0.144	NA
	1	23957309	6.83	0.141	NA
	1	24617505	6.03	0.142	oxidation resistance protein 1-like
	1	24880905	6.53	0.178	NA
	1	24881298	5.81	0.152	uncharacterized
	1	24881322	5.81	0.152	uncharacterized
	1	25149428	6.91	0.143	NA
	1	25149465	6.91	0.143	NA
	1	25149558	6.91	0.143	NA
	1	25724354	6.97	0.145	NA
	1	25817658	6.05	0.168	NA
	1	25817659	6.05	0.168	NA
	1	25817660	6.05	0.168	NA
	1	25817661	6.05	0.168	NA
	1	25822728	6.91	0.143	nuclear receptor coactivator 2-like
	1	25822852	6.91	0.143	nuclear receptor coactivator 2-like
	1	25822872	6.65	0.157	nuclear receptor coactivator 2-like
	1	25822888	6.91	0.143	nuclear receptor coactivator 2-like
	1	25827663	6.06	0.129	nuclear receptor coactivator 2-like
	1	25984157	6.91	0.143	uncharacterized
	1	25984434	6.02	0.158	uncharacterized
	1	26221696	6.16	0.128	rho GTPase-activating protein 190-like
	1	26221697	5.79	0.119	rho GTPase-activating protein 190-like
	1	26458728	6.91	0.143	NA
	1	26458755	6.91	0.143	NA
	1	27225046	6.1	0.124	NA

1	27372715	6.14	0.124	transient receptor potential cation channel subfamily M member 1-like
1	27372725	6.05	0.122	transient receptor potential cation channel subfamily M member 1-like
1	27937253	6.06	0.124	uncharacterized
1	27937383	6.06	0.124	uncharacterized
1	27937414	6.02	0.123	uncharacterized
1	28234691	6.05	0.123	NA
7	7251580	6.2	0.133	nucleolar MIF4G domain-containing protein 1-like

Significance threshold is 5.7 after correcting for the correct number of effective tests

Chapter 5: Conclusion and future directions

My dissertation investigated the biology and genetic basis of survival during acute low salinity (< 3) for the eastern oyster, *Crassostrea virginica*. The eastern oyster in an important aquaculture species along the Gulf of Mexico and mid-Atlantic coast of the United States. Oyster lines selectively bred for various environmental extremes are going to be important for the resiliency of the aquaculture industry moving forward. Specifically, enhanced survival during freshet events is crucial for coastal populations where pulse events and anthropogenic diversions of freshwater are common (reviewed in Du et al., 2021). Thus, understanding oyster physiology at acute low salinity and investigating the genomic regions underlying differential low salinity survival is important for breeding efforts moving forward.

Major conclusions from the low salinity challenges

Results from numerous extreme low salinity challenge experiments (seven total, two not explicitly discussed in this dissertation) provide a detailed picture of oyster mortality trends at acute low salinity. Low salinity challenge experiments were conducted across all three research chapters at either a constant temperature (27 – 28°C) or at a temperature that was manually adjusted to mimic ambient temperature, at two different times of year (spring and summer) and using oysters of various size-classes/age. The differences in animal size-class/age and time of year when challenges were conducted were not necessarily part of the experimental design, but more so a consequence of logistics (i.e. convenience of performing the challenge and what animals were available during that time). While challenges were not conducted at all possible combinations of age/size, time of year, etc., the information from the five challenges conducted in this dissertation could be structured into a decision-making table (e.g. Figure 1), where

breeders would be able to determine the appropriate low salinity challenge duration depending on the age of oysters, time of year, etc. This would be useful for breeding programs that wish to conduct the challenge and incorporate acute low salinity survival into their breeding efforts. However, it is important to conduct these challenges on oysters with a genetic background that is relevant to the breeding program or growing conditions, as the challenges in this dissertation were mainly conducted using selectively-bred lines adapted to growing conditions in the Chesapeake Bay (low salinity family lines and wild Choptank oysters; Allen et al., 2021). For example, if freshwater is a problem for aquaculture farms in Louisiana, then a challenge should be conducted using lines or wild diploids that are locally adapted to LA conditions to determine mortality patterns.

@ salinity 2.5, 27°C

	< 40 mm	> 40 mm
Spring	NA	2-4 weeks
Summer	4-9 weeks	2-4 weeks
Fall	2-3 weeks*	2-4 weeks*

* unpublished challenge data

Figure 1. Example decision making table to determine the exposure duration when conducting an extreme low salinity challenge at a salinity 2.5 & temperature 27°C depending on oyster size (< 40 mm or > 40 mm) and time of year (spring, summer, or fall).

Investigating low salinity tolerance and survival in spat/seed is a critical next step. Aquaculture farmers typically buy oysters as either spat-on-shell, larvae set on an oyster shell

(i.e. cultch), or seed, individual larvae typically set on a particle of ground oyster sell (i.e. micro cultch) for planting on their farm (Andrews and Mason, 1969; Hudson, 2019). Survival during low salinity exposure may differ substantially during the spat/seed life stage compared to later juvenile or adult stages. If oyster survival as a spat/seed is highly correlated to adult survival at low salinity, then breeding for either trait would theoretically result in the same genetic gains. However, low salinity survival would need to be treated as two separate traits when breeding if the genetic basis of survival differs between the two life stages. Breeding for low salinity survival in spat/seed oysters may be especially important for areas where farmers typically plant their new crop during the wet season.

The chronic (long-term, 6-month challenge) provides insight into the effect of temperature on survival in extreme low salinity. A relatively high temperature (27°C) was chosen for the challenges because mortality at salinity < 3 is negligible when temperature is < 20°C (Southworth et al., 2017; Chapter 3 chronic challenge), but a temperature of 24 – 25°C may have also been appropriate, with mortality likely delayed compared to higher temperatures. In the chronic challenge, mortality spiked first in early June at 25°C after 16 days above 20°C and again at the end of July after 27 days above 27°C (Chapter 3). While the magnitude of mortality was lower at 25°C compared to 27°C (Chapter 3), this may suggest a two-temperature threshold to extreme low salinity survival. It would be interesting to conduct side-by-side challenges, one at 25°C and one at 27°C, with individuals from the same families to see how family mortality at low salinity compares between the two temperature levels. Again, these challenges were conducted using oysters adapted to the growing conditions in the Chesapeake Bay, and populations native to other regions with different thermal ranges may exhibit differences in thermal tolerance at extreme low salinity. Regardless of population, is appears to be important to

keep the challenge temperature within ±0.5°C to maintain the dual-stressor. Temperature fluctuated slightly during the first three weeks of the short-term challenge, ranging from 24 – 27°C, which could have contributed to peak mortality occurring later in the challenge. In addition, temperature dropped to 22°C for 30 hours during a spring challenge (data not reported) and negligible mortality occurred over two months. There is no direct evidence supporting fluctuations in either temperature and/or salinity being responsible for delayed or negligible mortality described above, but fluctuations or slight decreases (3-5°C) in temperature may give oysters enough of a reprieve to survive extended periods at low salinity. Maintaining a consistent challenge environment (low salinity and high temperature) is very important for producing consistent mortality (repeatable phenotypes) over an expected timeframe under low salinity stress exposure.

Insight into the physiology associated with extreme low salinity stress

This dissertation proposes methods for measuring individual algae removal using a handheld fluorometer (chapter 3), which could be valuable tool for the broader scientific and aquaculture community. Smoothing splines captured the sigmoidal feeding trend for individual oysters, where individuals either depleted the algae quickly once feeding commenced or they slowly depleted the algae over a longer period of time. The family mean slope and midpoint of the splines for individuals sampled were marginally associated with the family mean survival in the chronic low salinity challenge cumulative survival (Chapter 3), but it is possible that other analyses or feeding metrics would better capture the feeding trends. For example, the onset of feeding may be more strongly associated with mean family survival at low salinity, where more tolerant individuals begin feeding more quickly after exposure to stressful low salinity conditions. Conversely, time to deplete all the algae may be more reflective of long-term low

salinity behavior and tolerance. However, in our experiments, some individuals did not deplete 50% of the starting algae concentration, so they would have a null (NA) value for this parameter. Converting this feeding metric into a binary phenotype (100% algae depletion or not) may produce a stronger association with low salinity survival compared to the average time to deplete 50% metric. In addition, from an application standpoint, measuring algae concentration at just two or three timepoints to determine if algae were depleted or not, would be easier to implement and allow for simultaneous sampling of more individuals on a given experiment day.

The results from the feeding experiment (Chapter 3) were intriguing and suggest, although only examined for a relatively small sample size, that feeding efficiency (speed of algae removal) is associated with low salinity survival. More experiments investigating the details of feeding, such as the activity of the cilia, control of gape/shell opening, characteristics of the exhalant siphon structure, and interfilamentary distance to the gill (mechansisms of feeding reviewed in Cranford et al., 2011), may provide an explanation for the observed differences in feeding behavior, as each structure and mechanism respond differently to stimuli. All oysters opened, to some extent, and conformed to the extreme low salinity in the challenge (Chapter 2), but differences in regulation of water, ions, or free amino acids may be responsible for differences in survival among individuals/families. Oysters must maintain a certain amount of free amino acids in order to function physiologically (e.g. Oglesby, 1981; Pierce, 1970; reviewed in Pourmozaffar et al., 2019), which would explain why all oysters remained slightly hyperosmotic in a salinity < 3 (Chapter 2). Osmolality of oysters in slightly higher and lower salinities could be investigated to determine at which low salinity level oysters are no longer able to fully conform. Thus, the concentration of a specific amino acid, for example, could be a molecular/physiological phenotype worth measuring. Additionally, investigating gene expression

using RNA-seq approaches during the challenge experiment, especially during the first week of exposure, could reveal pathways directly involved in low salinity survival and tolerance. An interesting next step would be to identify expression quantitative trait loci (eQTLs), which are regions of variation in the genome (genotypes) that explain variation in gene expression (Nica and Dermitzakis, 2013). Differences in gene expression could reveal a specific set of genes that are important during the molecular response to extreme low salinity exposure. Exploring physiology, gene expression, and eQTLs may help explain why mortality often spiked after ~ 8 days of exposure in our challenge experiments – what physiological limit are the oysters reaching? A biomarker or physiological assay (e.g. algae removal, regulation of ions) for low salinity stress would be helpful and, depending on the simplicity and ease of measuring, could provide a more precise phenotype for measuring low salinity tolerance.

Next steps for breeding of survival in extreme low salinity in oysters

Survival in acute low salinity has a strong genetic component. A moderate narrow-sense heritability was estimated for survival during the low salinity challenge using both a pedigree (Chapter 2 and 3) and marker-based (Chapter 4) relationship structure between multiple year classes and families of individuals. A moderate narrow-sense heritability suggests that this trait could be improved through selection, which is encouraging for programs wishing to incorporate acute low salinity tolerance into their breeding efforts. The presence of a potentially major effect QTL on Chromosome 1 (though this needs validation in additional populations) and the superiority of the BayesB genomic selection model (Chapter 4) suggest that initial genetic gains may be made by incorporating this (and possibly other) major-effect region. More substantial genetic gains may then be possible by implementing genomic selection considering the apparently polygenic nature of the trait.

This dissertation focused primarily on improvement of low salinity tolerance of oysters grown in floating gear typical of water column leases. Seed oysters, which are typically grown in bags and/or cages within or at the top of the water column, were used in all five challenge experiments. In contrast, oyster aquaculture in the Maryland-portion of the Chesapeake Bay is dominated by on-bottom culture using spat-on-shell (Hurley, 2021; van Senten et al., 2019). Freshet events will also likely impact on-bottom farms, but there may be other traits that are more important from a production standpoint. For example, traits such as shell thickness to avoid predators, ability to grow vertically to outcompete neighboring oysters for necessary resources, or the ability to survive heavy sedimentation may be more important for oyster survival on-bottom. Dedicating efforts to investigating traits important to on-bottom culture will further increase the productivity of the aquaculture industry.

For eastern oysters in the mid-Atlantic region, utilizing genomic selection (GS) for improvement of low salinity survival should be possible using the low salinity progeny test and a recently developed 66,000 Axiom SNP array. To breed for survival during exposure to freshet conditions (salinity < 3), a breeding program would first want to conduct a progeny test using similar conditions to the challenge experiments implemented in this thesis: exposure to salinity 2.5 and temperature 27°C for 1-2 months, depending on oyster size. To maximize genomic selection prediction accuracies, I would suggest that the experimental population consist of as many related individuals (e.g. half and/or full-siblings) as possible because the sample size (the size of the training population) and the relatedness between individuals in the testing and training population affect genomic prediction accuracies (e.g. Combs and Bernardo, 2013; Ehret et al., 2015; Habier et al., 2007; Wang et al., 2018). Survival (live or dead) should be recorded for all individuals in the challenge and tissue should be sampled and preserved for all individuals either

within 24 hours of death or at the end of the challenge for those remaining alive. Genotyping of all individuals should then be conducted using the 66,000 Axiom SNP array. In order to decrease genotyping costs and to potentially incorporate more individuals into the challenge, breeding programs could consider genotyping individuals at fewer markers (1,000 markers) if the experimental population consists of related individuals because there will be more linkage disequilibrium between markers and prediction accuracy should not be negatively affected (e.g. Kriaridou et al., 2020; reviewed in Zenger et al., 2019). Genomic selection training models can then be created, and validation approaches can be used to assess the accuracy of predicting an individual's survival based on their genome-wide markers. If the accuracy is moderate to high, which it was using F_2 families (Chapter 4), then broodstock oysters can be genotyped and the GS prediction model can be used to choose which individuals to spawn. The genomic selection model created in Chapter 4 may work for lines from the Aquaculture Genetics and Breeding Technology Center at the Virginia Institute of Marine Science and for oyster's native to the mid-Atlantic and Chesapeake Bay, but how the F_2 family structure affects model prediction remains unknown. If breeding for low salinity tolerance in other areas (e.g. Maine or Florida), the process above should be carried out and a different genomic selection model should be created because the genetic background may vary across populations.

Another important next step is to determine how low salinity survival in our challenge experiment correlates to survival in the field. This could be accomplished by creating families based on survival in the challenge experiment and then monitoring survival at a low salinity (< 3) field site. Challenges arise when comparing lab experiments to field experiments because there is a large amount of uncontrollable variability in the field (e.g. Cloern et al., 2017; Gilmour et al., 1997; Grodwohl et al., 2018). Lab experiments generally control for most ecological factors, but

other aspects of low salinity, such as reduced algae diversity and abundance, change in predation pressure, or presence of chemicals/toxins from freshwater runoff, may have a large effect on low salinity survival in the field. In addition, there is a great deal of variation from year to year in the field environment and poor discrimination (differences) in family mortality one year may not be reflective of actual differences in low salinity tolerance between families. Genotypes may respond differently to environmental variation (genotype-by-environment (GxE) interaction, Falconer and Mackay, 1996; Lynch and Walsh, 1998), where the best performing genotypes in one environment are not the best performing in a different environment. GxE interactions have been documented for various livestock and aquaculture species and can hinder the efficiency of selection if attempting to improve a trait across multiple environments (reviewed in Sae-Lim et al., 2016). Investigating the relationship between low salinity survival in the laboratory challenge and in the field, along with unveiling potential GxE interactions, is important if using the laboratory-based challenge to inform future breeding decisions.

The results from my dissertation highlight the potential of improving eastern oyster survival during acute low salinity exposure. A strong genetic component underlies low salinity tolerance, measured as a survival (binary) and day to death phenotype. However, future experiments are needed to fully understand the genetic architecture of the trait. Exploring additional omic-approaches (gene expression profiling and eQTLs), behavior, and physiology associated with survival during low salinity exposure are interesting next steps to better understand variable low salinity survival. The research findings, challenge experiment parameters, and algae removal methods are beneficial to the broader eastern oyster aquaculture community and can help improve the industry in the face of climate change.

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